

Can these agencies jell?

The plan to merge Japan's Science and Technology Agency with the education ministry is fraught with pitfalls but offers some opportunities.

Japan's Prime Minister, Ryutaro Hashimoto, has met remarkably little opposition to his plan to merge the Science and Technology Agency (STA) and Monbusho, the ministry of education, science, sports and culture. That is surprising considering that the two organizations are about as compatible as oil and water. What is more, leaders of government research organizations throughout Japan are aghast at the manner and speed at which the plan has moved ahead (see page 327). But they have had no say in the decision.

The merger is very much the brainchild of Hashimoto, who was infuriated by the STA's failure to deal with a string of accidents at the Power Reactor and Nuclear Fuel Corporation and who quickly killed more sensible attempts to upgrade the agency into a ministry. There was no discernible opposition to the move in Hashimoto's reform council, which has only one scientist — Akito Arima, president of the Institute of Physical and Chemical Research (RIKEN), who seems to have taken an uncharacteristically ambivalent stance on the proposal. Moreover, the merger has gone largely unnoticed by the public and media, who have focused their attention on Hashimoto's difficulties elsewhere.

Yet the merger of the STA and Monbusho is the most radical reform of Japan's public sector science system in decades. The two organizations could hardly be more different. The science and technology section of Monbusho is tucked away in a dark corner of one floor of the ministry's six-storey building. The ministry's brightest career bureaucrats spend at most a couple of years in that section before moving on to the ministry's much stronger sections in education. The STA, on the other hand, is the only government agency devoted entirely to science and technology.

Some proponents of the merger argue that STA officials will bring much-needed new blood to the education ministry and help to break the ministry's notoriously bureaucratic stranglehold on the university research system. But even if all STA officials move to Monbusho — which is unlikely, given that some sections of the agency are to be hived off elsewhere — they will be greatly outnumbered. Furthermore, the problems in the university research system are caused as much by a sclerotic combination of conservatism, favouritism, deadwood and inbreeding in the universities themselves as by the policies of Monbusho bureaucrats.

The potential pitfalls of the merger are many. One particularly dangerous idea is that the Institute of Space and Astronautical Science (ISAS) under Monbusho should merge with the STA's much bigger National Space Development Agency. The acute danger in that apparently rational approach is a loss of the virtues of ISAS, a remarkable organization that has shown the world how to run a dynamic worldclass space science programme on a shoe-string budget.

So what benefits can be hoped for? The STA does have a greater awareness of the need for objective research assessment and may, with sufficient determination, be able to introduce such practices in universities that so far have insisted on 'self-evaluation'. Furthermore, the STA has, through a 'hands off' approach, allowed RIKEN to become one of the most successful enterprises for basic research in Japan. The merged organization should apply the RIKEN model to university research institutes, in particular those for joint university use, such as ISAS and the National Astronomical Observatory — which could bring much-needed autonomy to these organizations and let them make better use of limited funds. □

Nuclear debate required

It is time for France's National Assembly to flex atrophied nuclear muscles.

The United States has one sort of cheese but 366 designs of nuclear reactors; France has 366 sorts of cheese but just one design of reactor. So goes a favourite quip used by French officials to express their pride in the meticulous planning that has produced the world's foremost nuclear power programme, the source of 80 per cent of the country's electricity. But the coherence of the programme increasingly resembles a lump of gruyère.

The reason is that France has continued doggedly to pursue the plutonium cycle that has dominated nuclear thinking since the 1970s. But the dream of a generation of fast-breeder reactors that would burn plutonium and breed further fuel has evaporated in the face of economic and technical problems, while plutonium is increasingly recognized to be dangerous environmentally and for proliferation. The root cause of France's unreasoned passion for plutonium is that influential technocrats and lobbyists have been able to make decisions in a system that has effectively shielded them from direct public scrutiny and democratic control.

A shift in this balance of power is now being brought about by

the Socialist government of Lionel Jospin elected last June, and the influence of its electoral allies the Greens. By deciding to unplug Superphénix, a FF60 billion failed commercial prototype fast-breeder, it dealt a sharp blow to the nuclear lobby. Superphénix was symptomatic of the French disease whereby long-term strategies that are difficult to reverse once in place are decided upon prematurely without proper debate. Last week's decision by a large group of a new generation of politicians to challenge the goals of the national nuclear waste disposal programme (see page 322) marks a step towards recovery.

Their opposition to the current excessive emphasis on deep storage can only be described as good sense, as this emphasis is resulting in neglect of other research avenues that may be more promising in the long term. Prudence is all the more desirable given that the choices that will be made over the next few years will fix French nuclear waste policy for decades. Also needing scrutiny is the wisdom of using MOX (mixed uranium/plutonium oxide fuel) in reactors. In short, the National Assembly should convene a national debate on the entire nuclear power programme. □



Closure of primate lab angers both researchers and critics

[WASHINGTON] The best known primate research centre in the north-eastern United States is to close at the end of December. The move leaves researchers in New York with no convenient access to primates, and animal rights activists still angry over the shipment of half the former chimpanzee population of the Laboratory for Experimental Medicine and Surgery in Primates (LEMSIP) to a facility operated by the Coulston Foundation in New Mexico.

New York University (NYU) pledged to find retirement homes for LEMSIP's remaining 28 chimpanzees after students occupied the office of Jay Oliva, the university's president, overnight on 10 November. But last week NYU informed staff at LEMSIP that the facility will close for good on 31 December, after negotiations for its purchase by the Aaron Diamond AIDS Research Center had broken down.

According to sources close to the negotiations, NYU had wanted \$1.1 million for the facility, located at Tuxedo, 40 miles north-west of New York. But Aaron Diamond was only prepared to pay half this sum.

The closure marks the end of a saga that began two years ago, when NYU sacked Jan Moor Jankowski, the then director of LEMSIP, and said that it would transfer the facility and its animals to Coulston (see *Nature* 376, 543; 1995).

Aaron Diamond had been negotiating to buy the facility when the Coulston deal was announced. The AIDS research centre sub-



Home from home: the Coulston Foundation in New Mexico, to which chimpanzees have been moved.

sequently severed its affiliation with NYU, and most of its researchers — including David Ho, its director — left NYU to take up faculty positions with Rockefeller University in New York.

Animal rights activists, who have long accused Coulston of mistreating chimpanzees, attacked the proposed transfer. And Jankowski sued NYU for \$17 million, alleging that he was being persecuted by the university for “whistle-blowing” on another researcher's work, which he considered unethical (see *Nature* 382, 746; 1996). The case is still being considered by a New York judge.

Although NYU and Coulston failed to agree terms for the transfer of LEMSIP itself, 100 chimpanzees were transferred to Coulston's New Mexico facility and another 100 were retired, leaving the current population of 28 animals. Meanwhile, Jankowski and

other researchers at LEMSIP have formed an informal alliance with animal rights activists, including groups that oppose all animal experimentation, to attack NYU's management of LEMSIP.

“When I was approached by anti-vivisectionists and they wanted to know what I was doing, I'd let them know that it was important research,” says Jankowski. Disagreements about animal treatment “can be solved amicably,” he adds. “It doesn't have to be all about barbed wire and security guards.”

LEMSIP's closure will mean that there is no primate research facility left in the New York area. Preston Marx, a virologist at Aaron Diamond whose recent work includes confirmation that the use of progesterone implants can speed transmission of HIV-like viruses between macaques (see *Nature Medicine* 2, 1084; 1996), is now arranging to transfer his 140 macaques to a primate facility elsewhere in the United States.

Marx, a staff researcher at Aaron Diamond and a professor at NYU, plans to stay in New York temporarily after vacating his laboratory at LEMSIP, but will probably have to move to another part of the country to continue his work. “This is a great loss for the New York area,” he says.

After the 10 November protest, Robert Berne, NYU's vice-president for academic development, promised that half of the remaining 28 chimpanzees would be retired this year and the rest later on, and that none would go to Coulston. He also said that Oliva would ask the senate academic affairs committee to “assess the desirability” of putting more students on the university's animal care committee.

But Berne says that the remaining chimpanzees were all going to be retired anyway. “I don't think we backed down at all,” he says, adding that the protesters left Oliva's office because otherwise “they would have been expelled from the school.”

Colin Macilwain

Gingrich urges doubling of health funds

[WASHINGTON] Newt Gingrich, the Speaker of the US House of Representatives, last week joined a growing band of prominent politicians calling for a doubling of government spending on biological and biomedical research.

Addressing a meeting in Georgia on women's health issues, Gingrich, the senior Republican in the House, said that such a move would contribute towards “the explicit goal of making the United States the primary producer of health care information in the world”.

But, he added, reaching that goal should include a “dramatic overhaul” of the National Institutes of Health (NIH), which he said should be more transparent and more aggressive in using information technologies to publicize its work. “People ought to be able to get information faster and more openly,” said Gingrich.

“I want the National Institutes of Health to become much more of an Internet and information-age-oriented system with a total transparency so that people can know what's being applied for, what's available, what kind of research is going on.”

Gingrich said that a maximum of 18 months should elapse before doctors are told about new treatments and drugs. He proposed an Internet site continually updated to give push-button access to new scientific and medical information.

Diane Wax, director of legislative activities at NIH, says the agency is already making more medical information available to doctors and patients through the Internet. “We're doing much more than he's aware of.” But, she adds, with millions of people not owning computers, “we can't make the Internet the only source of information for the public”.

Meredith Wadman

Nuclear debate splits open in France...

[PARIS] A 15-year programme set up by France in 1991 to explore options for the disposal of nuclear waste has come under attack from a large group of politicians, who claim that the programme has become biased in favour of just one option, geological storage.

The group also says that the programme pays insufficient attention to long-term alternatives, such as research into the transmutation of long-lived nuclear waste into shorter-lived elements.

The reopening of the debate about nuclear waste comes less than six months after the Socialist government decided to abandon the fast-breeder reactor Superphénix, removing the rationale for the continued pursuit of the plutonium fuel cycle, and with it the need for the world's largest reprocessing plant at La Hague on France's northern coast.

Taken together, these events suggest the overall strategy of France's nuclear power programme is being exposed to extensive national scrutiny for the first time. "I am very satisfied about this," says Claude Detraz, head of the Institute for Nuclear and Particle Physics at the Centre National de la Recherche Scientifique (CNRS).

The nuclear waste programme was established by a 1991 law which postponed a decision on what to do with waste until 2006, to allow time for more research into three approaches to disposal. One is to develop

techniques for transmuting radioactive elements in waste. Another is to study conditions for surface storage. The third requires the government to select two sites for the construction of deep 'rock laboratories'.

But an appeal launched last week, and already signed by more than 40 politicians belonging to the governing Socialist coalition, protests that the deep storage approach is already being favoured at the expense of the others — more than half the FFfr1 billion (US\$173 million) annual budget of the waste programme currently goes on studies on deep storage.

"The thinking of the nuclear lobby [in favouring deep storage] is 'out of sight, out of mind'; they want to give people the impression that the problem of waste has been solved when it has not," says Michèle Rivasi, the newly-elected member of parliament leading the protests.

More broadly, the appeal reflects an emerging preference for 'reversible' options where waste would be stored in a form that could be retrieved for processing should new techniques become available. Claude Allègre, the research minister, recently said that he opposes deep storage as "dangerous for future generations".

The appeal also demands the reopening of public inquiries into the siting of the planned rock laboratories. It points out that one site in la Vienne department approved by public inquiry had been previously



deemed unsuitable on geological grounds by the independent National Evaluation Commission (CNE) responsible for monitoring the waste programme.

The industry ministry last week agreed to shift 15 per cent of funding for deep storage to reversible surface storage options. Meanwhile, the Socialist group in the National Assembly has decided to hold a debate on the entire nuclear power programme in the next few weeks.

The politicians' call for greater emphasis on fundamental research into waste disposal options echoes a report released earlier this year by the CNE, which highlighted, for example, a virtual absence of research on the disposal of dangerous fission products such as iodine and caesium.

Nonetheless, Bernard Tissot, who chairs the CNE, argues that the organization of French research on nuclear waste has "improved considerably" since the release of a highly critical report by his commission in 1995 (see *Nature* 376, 204; 1995).

For example, the GEDEON joint body to coordinate such research has been set up by the CNRS, the Atomic Energy Commission and Electricité de France (EDF), while CNRS itself last year set up a FFfr5 million multidisciplinary programme (PACE) on nuclear waste disposal, a fivefold increase on previous spending.

GEDEON intends to fund a small FFfr2-million experimental model of a novel subcritical reactor for the transmutation of waste proposed by Carlo Rubbia, former head of the European Laboratory for Particle Physics, CERN. "We are too locked into existing technologies," says Detraz, who argues that the use of fast-neutron reactors for incinerating plutonium (see *Nature* 365, 381; 1993) is also worth exploring.

Detraz says that the funding of such research is all the more urgent because of the long delays in introducing large-scale systems; it takes 10 years to develop and test a demonstrator and more than 20 for a prototype.

Declan Butler

... as new waste body is proposed for the UK

[LONDON] The management of radioactive waste in the United Kingdom should be taken over by a new commission, independent of the nuclear industry and answerable to government and parliament, says a report by Britain's Parliamentary Office of Science and Technology.

The body would be funded through a levy on the nuclear industry, and would be responsible for selecting a waste disposal site. It would also carry out research, which would be peer-reviewed before publication. Its decision-making would be open to public scrutiny, and it would take into account the concerns of "all stakeholders".

The report says that a separate organization should

then take over the job of designing, building, financing and operating a waste disposal facility approved by the commission and regulatory bodies. The work would be done under contract to the government and the nuclear industry.

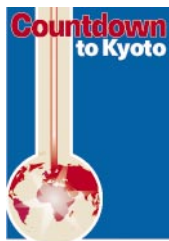
The proposals would mean breaking up Nirex, the company owned by the nuclear industry that is at present responsible for all aspects of radioactive waste disposal. Nirex was recently prevented by the government from further investigations on a proposed deep underground repository for waste from nuclear power stations (see *Nature* 386, 423; 1997).

That decision led to widespread agreement that radioactive waste

management needs a thorough review before a further site is found and that Nirex may not be the best organization to take the issue forward. Nirex has been criticized for a lack of openness in the past, and some view it as being too close to the nuclear industry.

The timing of a government decision on nuclear waste disposal policy is likely to depend on the outcome of a review of radioactive waste management announced this week by the House of Lords Select Committee on Science and Technology. The committee's final recommendations will not be presented to parliament until next summer. But Nirex has already shed almost half of its 200 staff. **Ehsan Masood**

Australia plays hard to get



[SYDNEY] The Australian government, squeezed between the country's mining industry and the international community, has announced measures to curb greenhouse gas emissions at a cost of A\$180 million (US\$124 million) over four years. But the planned shift towards renewable energy aims not to cut emissions or even to level them out, but to reduce from 28 per cent to 18 per cent the anticipated increase over 1990 levels by the year 2010 — a reduction of 39 million tonnes of carbon a year.

Supported by Australia's large exporters of coal and minerals, the Prime Minister, John Howard, has refused to yield to pressure from other countries to accept mandatory emissions limits in the run-up to the Kyoto meeting next month. He claims the European plan for reducing emissions by 15 per cent by 2010 would cost Australia 90,000 jobs.

Howard told parliament that Australia wants to retain "bargaining chips" and will

not "telegraph its negotiating position".

But the planned measures have not had an enthusiastic reception from others. Mark Diesendorf, vice-president of the Sustainable Energy Industries Council of Australia, described them as an "overrated voluntary scheme to assist business to make cost-effective reductions they should already be doing".

Following the government's controversial axing last May of its Energy Research and Development Corporation, which made grants for research on energy conservation and alternative energy, the new package does not include any grants for research. This has precipitated the resignation of Ros Taplin, director of Macquarie University's Climate Impact Centre in Sydney, saying there were no prospects for funding its research.

This week it was revealed that, in formulating a position that continues to generate controversy at home and abroad, Howard had not sought formal advice from Graeme Pearman, the leader of the country's largest programme of climate research at the Commonwealth Scientific and Industrial Research Organization.

Peter Pockley

Indian PM under fire on Edinburgh deal

[LONDON] One of India's main environmentalist groups has criticized the prime minister for agreeing to make "significant reductions" to greenhouse gas emissions after the Kyoto conference.

The prime minister, I. K. Gujral, signed a declaration to that effect along with other Commonwealth leaders at summit meeting in Edinburgh at the end of October.

But Anil Agarwal, director of the Centre for Science and Environment in New Delhi, says Gujral should not have signed. Developing country participation is one of the US conditions to a greenhouse gas reduction treaty. Agarwal says that agreeing to the condition without asking for anything in return has left India and other developing countries with nothing to bargain with at Kyoto.

"The Indian prime minister may have jeopardized the position of the South even before the conference got under way," says Agarwal. "Gujral's endorsement also puts in jeopardy any demand to fix emissions on a per capita basis, rather than a per country basis."

Ehsan Masood

Canada's line dents its green image

[MONTREAL] Canada, the last of the G7 group of industrialized nations to announce its targets for next week's Kyoto meeting on global warming, has proposed a reduction of carbon dioxide emissions to 1990 levels by 2010. The proposal is in line with that of the United States — apparently confirming Canada's transformation from one of the world's politically 'greenest' countries to one of the most environmentally cautious.

Canada's target is less than that agreed five years ago at the signing of the climate convention in Rio, namely a return to emissions at 1990 levels by 2000. Only Quebec has promised to adhere to the terms of the climate convention. The province has been more successful than others in reducing greenhouse gases, largely because of its reliance on hydro-electric power.

Canada's past support for environmentalist causes helped secure the country two high-profile prizes. Elizabeth Dowdeswell, former junior environment minister, was picked four years ago to head the United Nations Environment Programme. And two years ago Montreal was picked to host the secretariat of the UN biodiversity convention. Canada's shifting politics has coincided with the departure of Dowdeswell from the UNEP. There is also speculation that her replacement, the German Klaus Töpfer, may want all of the UN's environment agencies to move to Bonn. **David Spurgeon & Ehsan Masood**

Wirth hands over leadership of US delegation

Is this the way out? Wirth (right), who is leaving the US State Department, in discussion with the UK's Michael Meacher.



[LONDON] US President Bill Clinton has picked Stuart Eizenstat, a senior State Department official and former domestic policy assistant to President Jimmy Carter, to lead the US delegation to the Kyoto climate talks.

Eizenstat, a lawyer by training, is under-secretary of state for economic, business and agricultural affairs. He replaces the expected choice, Timothy Wirth, under-secretary of state for global affairs, who announced last week he was leaving the

department to head the United Nations Foundation, set up to manage Ted Turner's \$1 billion donation to the United Nations.

Wirth's announcement at this critical stage is unlikely to raise confidence in the prospects for a greenhouse gas reduction treaty.

The United States has proposed stabilizing emissions at 1990 levels, but on condition that developing countries contribute to emissions reductions. Developing countries oppose this. Unless one side shows

flexibility, a treaty is unlikely.

Wirth had been carefully guiding US strategy leading up to Kyoto. An important element has been to downplay the prospects of a radical agreement at Kyoto. Wirth has travelled to major capitals aggressively promoting the US line. But, as an experienced politician with a well known commitment to environmental causes, his comments were seen by some as an attempt to avoid the United States being blamed for a failure to reach agreement.

E.M.

Ethical terms set for breast cancer test

[WASHINGTON] A team that has just been granted British patent rights on a gene whose mutations are linked to breast cancer has licensed the use of the genetic data in an unusual agreement that requires the exclusive licensee to meet strict conditions for what the team views as ethical use of any diagnostic tests it develops.

These include broad sublicensing of diagnostic tests to other concerns, a requirement for pre- and post-test counselling, and a ban on direct advertising of screening tests to the public. The use of the techniques will be made freely available to Britain's National Health Service (NHS).

The London-based Cancer Research Campaign Technology (CRCT) and its US partner, Duke University in Durham, North Carolina, have been granted a British patent for the full-length coding sequence of the gene, thought in its unmutated state to produce a tumour suppressor protein.

But the US patent situation remains open. Both CRCT/Duke and their major competitor in the race to find the gene — Myriad Genetics of Salt Lake City, Utah — have applied for *BRCA2* patents. Myriad announced its move the day before the CRCT/Duke team published its discovery of the mutation in 1995 (see *Nature* 378, 789; 1995). A decision by the US Patent and Trademark Office is not likely for at least two years.

CRCT/Duke's rights under the British patent include pharmaceuticals, diagnostic methods and a method for producing the protein manufactured by the gene. The US company Oncormed of Gaithersburg, Maryland, has been granted an exclusive worldwide licence to the patent for diagnostic services and products.

In return, Oncormed has agreed to sublicense diagnostic tests broadly to hospitals, nonprofit organizations and commercial groups. As a condition of receiving the licence, it has granted the UK NHS a free licence to practise *BRCA2* testing. "Our goal is to broadly license gene discoveries for diagnostic purposes," says Leslie Alexandre, Oncormed's corporate affairs vice-president.

One author of the *Nature* paper, Mike Stratton, professor of cancer genetics at the Institute of Cancer Research, Sutton, says that his group's intention in patenting *BRCA2* was to prevent it from becoming the exclusive province of one commercial interest. Taking out the patent was "the only way of defending the gene" and of having "some control over its future," he says.

In choosing to grant an exclusive licence to Oncormed, he says, CRCT set down four principles that the company promised to adhere to: that it would sublicense diagnostic tests to other concerns; that access to diagnostic tests should be only through doctors,

with thorough pre- and post-test counselling; that no attempt should be made to "drum up business" through advertising to the public; and that the NHS, which contributed to the gene's discovery, should not be charged a licence fee.

Patricia Murphy, a geneticist at Albany Medical College in New York state and a consultant to Oncormed, says that to her knowledge the arrangement is the first of its kind. Typically, she says, "the reason you get an exclusive licence is to capture the market and prevent others from doing the testing".

But some say that agreements with private companies cannot be substituted for government regulations requiring that genetic testing be conducted ethically and responsibly. One critic is Neil Holtzman, the director of genetics and public policy studies at the Johns Hopkins Medical Institutions in Baltimore, Maryland.

Holtzman chaired the task force on genetic testing of the Working Group on the Ethical, Legal and Social Implications of Human Genome Research, established by the US National Institutes of Health and the Department of Energy. "I would not entrust a company, as apparently CRCT has done, to ensure that all testing will be appropriate," says Holtzman. "I think you need external control and review."

Stratton's group once collaborated with Myriad, but fell out with it in 1994 when Myriad filed for a broad patent on the other breast cancer gene, *BRCA1*, which its scientists discovered in collaboration with researchers at the University of Utah. CRCT and Stratton say that they have profound philosophical differences with Myriad about how widely available the diagnostic and therapeutic applications of patented genes should be made.

Meredith Wadman

NSF hires expert to tackle 'millennium bug'

[BOSTON] Faced with the possibility of widespread computer failures on 1 January 2000, the US National Science Foundation (NSF) has hired an expert to help the researchers it funds to get through the millennial transition with minimal disruption.

Mark Haselkorn, a professor of technical communication at the University of Washington in Seattle, has been appointed to determine how ready researchers are to cope with the year 2000 'bug' and to formulate strategies to address potential difficulties. The problem stems from the fact that many computer programs and chips represent years by two digits rather than four and cannot accept that the year '99' is followed by '00'. So operations based on two-digit years are likely to be fouled up.

Steve Williams, a director in the information systems division at NSF, says that, although responsibility for addressing the issue rests with grant recipients and their institutions, "the problem appears serious enough for us to offer technical assistance". He admits that the initiative is unusual for NSF, "since we normally take a hands-off approach with our grantees".

Haselkorn will not be involved with NSF's internal efforts to make sure its own systems function properly. He says his first task will be to conduct a study of user communities, with protecting data the chief objective. Priority will be given to ensuring that NSF facilities, such as telescopes and Antarctic research centres, avoid serious malfunction.

The second set of targets will be large NSF-funded research centres at universities and other institutions. Next, Haselkorn will

try to assist individual researchers. "There's no way I can help every one of these people," he says. "But I hope to make information available on the web and by other means so they don't feel totally clueless."

Researchers who share data with others must ensure that changes in other databases will not disturb their own databases, says Haselkorn. They also need to figure out whether their systems are compliant. "Even if the system is capable of handling four-digit dates, that doesn't mean it's been programmed to actually use them," he says.

An important part of his mission, he adds, will be to "show how resources can be spent more efficiently, with less effort devoted to things that are unlikely to work". Highly interconnected systems, with "data shared all over the place", are the most problematic.

"In those cases, even if you could do your own fix, the odds of other parts of the system being fixed on time are vanishingly small," Haselkorn says. "So you might want to take a different strategy, thinking instead about how to mitigate the impacts."

Haselkorn is chairing a committee charged with writing a technical information statement on the issue for the US Institute of Electrical and Electronics Engineers. The report is due for release in January 1998.

NSF hopes that Haselkorn will help to dispel some of the myths and hype surrounding the year 2000 problem. "We're trying to alleviate fears that this will be a major catastrophe, and instead take steps to deal with the issue in a level-headed way," Williams says.

Steve Nadis

Lift spending cap or lose Mars mission, ESA panel warns

[MUNICH] Europe's space ministers have been told that a planned mission to Mars — known as Mars Express — will almost certainly have to be abandoned unless they lift a 'cap' placed two years ago on the science budget of the European Space Agency (ESA).

The warning came last week from members of ESA's Science Programme Committee (SPC). It forms part of a resolution outlining the consequences of the budget cap imposed at a ministerial meeting in 1995. The cap has already led to a 9 per cent reduction in the budget of the science programme.

The resolution also warns of the threat to the concept of large 'cornerstone' missions, the backbone of ESA's long-term science plan, Horizons 2000. The agency could be left unable to plan its space science strategically, and hopes for a new cornerstone mission in fundamental physics would be dashed.

"Europe will have to restrict its space programme largely to niches left by other agencies," says the resolution. As a result, it would "lose the supranational leadership and visibility" which allowed it to emerge on the world scene.

The resolution endorses concern expressed earlier this month by ESA's Space Science Advisory Committee (see *Nature* 390, 8; 1997). As the SPC is made up of delegates from member states, it is closer to ministers than the advisory committee, whose 11 members are independent space scientists.

Nevertheless few feel that the programme committee is likely to influence the course of financial stringency on which Europe's space ministers seem set, particularly as any decision to restore full index-linking of the ESA budget would require unanimous support from member states. Several — including Germany, Britain and Sweden — remain opposed to lifting the funding limit.

But Johann Bleeker, director of the Dutch Space Research Organization, and one of his country's delegates to the SPC, hopes that an awareness that Europe's international status in space efforts could suffer if it abandons its independent mission to Mars will shake ministers out of their stubbornness.

One piece of good news was announced at the SPC meeting last week. After several years of negotiations, ESA has signed an agreement with the Russian Space Agency that Integral, the gamma-ray observatory, will be launched on a Russian Proton launcher on schedule in April 2001.

In exchange for the launch, Russian astronomers will have access to about a quarter of Integral's observing time. Integral was approved as the next ESA medium-sized mission in 1993.

Alison Abbott

NASA told to make greater use of 'cheaper' private data

[WASHINGTON] The US Congress last week gave the National Aeronautics and Space Administration (NASA) permission to build and launch a quick replacement for an ocean wind monitoring instrument lost when a Japanese satellite failed in orbit in June. But one member of Congress has accused NASA of not checking if the satellite data could be bought for less money.

NASA will spend \$93 million to fly its Quick Scatterometer (QuickSCAT) mission in November 1998. It will help to make up for the loss of a NASA-funded sensor on Japan's ADEOS Earth science satellite (see *Nature*, 388, 105; 1997), which was to have provided data until the more capable SeaWinds is launched on ADEOS-II in 2000. Ocean wind data help predict the movement of tropical storms and are used in climate research.

QuickSCAT will be the latest experiment in cheap, fast space missions, with extremely short development and streamlined procurement of an 'off-the-shelf' satellite. The instrument itself will be built from SeaWinds spare parts to keep costs down.

Earlier this month, Dana Rohrabacher (Republican, California), chairman of the House of Representatives subcommittee for space, wrote to the NASA administrator, Daniel Goldin, praising him for picking a cheap, fast microsatellite for the job.

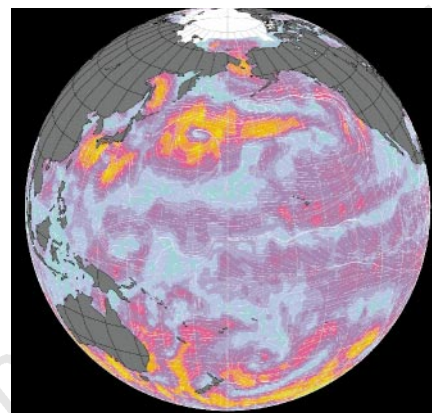
But he also claimed that "the commercial sector could provide this data immediately from existing satellite data streams". Buying a new satellite, he suggested, would therefore be a waste of money.

Rohrabacher declined to name companies that could provide the service. And last week Goldin told James Sensenbrenner (Republican, Wisconsin), chairman of the House Science Committee, that NASA knew of no commercial source of ADEOS-type sea surface wind data.

The compromise is for the agency to go ahead with QuickSCAT while issuing a formal request for data from commercial vendors. "If such data were available," Goldin wrote, "it could have the potential to achieve cost savings and/or the added benefit of a backup source of data if a problem were to arise with the QuickSCAT mission."

Rohrabacher, Sensenbrenner and others in Congress accuse NASA of dragging its feet on buying science data, particularly Earth remote sensing data, from private companies. But NASA science managers say — sometimes under their breath — that commercial data rarely meet their needs.

Congress, particularly the House Science Committee, has forced the issue by giving NASA \$50 million for commercial data pur-



It's windy down there: ocean surface winds over the Pacific seen from a NASA Scatterometer.

chase in 1997, money that is so far unspent. NASA has also been ordered to report by next spring on "the extent to which the baseline scientific requirements of Mission to Planet Earth [the Earth science programme] can be met by commercial providers".

Last week, NASA showed some movement by announcing that 11 companies would receive up to six months funding to analyse and validate data products that might fit government scientific needs.

The agency also received encouraging news recently on another project to foster partnership in remote sensing between government and industry: the proposed LightSAR imaging radar satellite. Congress gave NASA \$12 million last year — money it had not requested — to begin planning for a satellite to take synthetic aperture radar (SAR) images of the Earth from orbit.

Such imagery can be used for studying crustal deformation, tracking the motion of ice sheets and examining ocean circulation patterns. At the same time, SAR is believed to have commercial applications from topographic mapping to crop monitoring.

The idea of LightSAR is that industry and government would share the estimated \$150 to \$250 million bill more or less equally. Last March, NASA asked four companies to study if such a partnership was commercially attractive. The preliminary answer, says LightSAR pre-project manager Steven Bard of the Jet Propulsion Laboratory, is yes.

"All the teams found that there is a very high market potential," he says, though he adds the market is also uncertain. Results will be presented to managers at NASA headquarters in January and mean there is a "good chance there will be a meaningful investment" by industry in LightSAR. If so, it would increase the chances of winning significant government funding in 1999, with a launch three years later.

Tony Reichhardt

'Better adherence vital in AIDS therapies'

[WASHINGTON] Combination therapies for HIV-positive patients will not help certain high-risk groups unless researchers can rapidly improve their ability to persuade patients to follow the complex drug regimens involved, a meeting in Washington DC was told last week.

The Forum for Collaborative AIDS Research, a public-private-sector partnership group which helped to organize the meeting, is to prepare a 'research agenda' for the US National Institutes of Health (NIH) and drug companies, proposing ways in which biomedical and behavioural scientists can cooperate to achieve that goal.

Gerald Friedland, head of the AIDS programme at Yale University School of Medicine and co-chair of the meeting, described patient adherence as "perhaps the greatest challenge" to combination therapies. "Adherence has now emerged as the Achilles' heel" of such therapies, he said; improving it would be "a daunting task".

Since early 1996, when researchers established that aggressive treatment with combinations of antiretroviral drugs could successfully suppress HIV levels and thus prevent the onset of full-blown AIDS, the therapies have been widely prescribed in the United States. As a result, the number of new AIDS cases has already fallen sharply.

But the therapies require patients to follow arduous regimens, taking up to ten pills a day at fixed times. Data from clinical trials indicate that a treatment's effectiveness can be permanently undermined by even

brief non-adherence. Failure to take the drugs at the right times, even for a few days, can lead to restoration of the viral load to previous levels — and with a virus resistant to the therapy being used.

Scientists and advocacy groups also fear that such non-adherence might generate HIV strains resistant to all available combination therapies. But data on such an outcome are sparse. The question "isn't being looked at systematically", says Friedland. "We don't yet know if such resistant strains will emerge," he says, "but nothing indicates that it will not happen."

The meeting, co-sponsored by the National Minority AIDS Council and NIH's Office of AIDS Research (OAR), heard that existing knowledge on patient adherence was of little help to AIDS researchers. Most therapies are insensitive to minor lapses, so ensuring strict adherence has been a low research priority.

The research agenda, to be published early next year, is likely to suggest research work in three main areas of interest, officials at the Forum and at OAR said: techniques for measuring patient adherence, factors that predict patient behaviour and interventions to change that behaviour.

Options for measuring adherence include technology-based solutions, such as drug-bottle caps that electronically record whenever the bottle is opened, as well as self-reporting by the patient, although this is known to have flaws.

Although it is sometimes assumed that

the high-risk groups now being targeted for combination therapies, including drug addicts and homeless people, will not adhere to complex drug therapies, this assumption is not well supported by existing data, and more research is needed to identify the factors that influence adherence.

Interventions to encourage adherence can range from education and family support to cash incentives, which have been tested with some success on homeless people in San Francisco. But again, the meeting heard, little is known about which approaches are most effective.

As well as identifying necessary research in the three areas, the research agenda will prioritize the activities as immediate, medium-term and long-term, and suggest which government agencies or drug companies are best placed to pursue each one.

Officials at the Office of AIDS Research, which coordinates the \$1.5 billion AIDS research programme at NIH, pledged to follow through on the research agenda. "We did a poll across the NIH institutes in the summer to see what they were doing about adherence and we discovered there is still not very much going on," says Judith Auerbach, chair of OAR's behavioural and social science coordinating committee.

Work to study patient adherence is already under way at several NIH institutes and more is being supported by OAR's new \$7-million-a-year Prevention Science Initiative, Auerbach says.

The urgency of investigating adherence has been raised by recently published studies indicating that combination therapies will never eliminate the virus, so that patients must stay on the complicated therapies indefinitely to prevent the onset of AIDS.

Some speakers at the meeting questioned whether patients could ever adhere to such complex therapies. "Imperfect adherence is a medical fact of life that should be accepted," said Charles Flexner, a pharmacologist at Johns Hopkins University Medical School in Baltimore, Maryland. Flexner predicted that better drugs, with far simpler regimens, will become available within two years.

"I have no doubt that we'll see more friendly drug regimens in a couple of years," says Tony Fauci, director of the National Institute for Allergy and Infectious Diseases, the NIH institute that supports most AIDS research. "But that doesn't rule out the need to understand adherence, or the lack of it, in individuals."

William Paul, director of the Office of AIDS Research until his return to research at the end of last week, says that more research into adherence "would be a small cost and, if we can make some headway it might be very valuable".

Colin Macilwain

Sub-Saharan Africa hit hardest by epidemic

[WASHINGTON] While the United States works to contain AIDS with sophisticated combination therapies (see above), developing countries are being ravaged by the epidemic more rapidly than ever, according to the United Nations AIDS programme (UNAIDS).

According to UNAIDS' annual survey, released this week, 5.8 million people were infected with HIV this year, while the total number of adults infected was just under 30 million — one in a hundred of the world's adult population.

The epidemic remains overwhelmingly centred on sub-Saharan Africa. UNAIDS has revised its previous estimates for the region



UNAIDS: HIV cases up again.

we know about the AIDS epidemic, the worse it appears to be," says Peter Piot, executive director of UNAIDS. "In sub-Saharan Africa, the situation is even more desperate than had been thought." More than 20 million people, or 7 per cent of the adult population, are now infected with HIV in sub-

sharply upwards by using new models that take into account the differing pattern of the epidemic in different countries.

"The more

Saharan Africa, UNAIDS says. Worldwide, 2.3 million people will die of AIDS this year.

UNAIDS has also revised upwards the estimates it published last year: after the adjustment is taken into account, the number of new infections rose 9 per cent this year from last, and the total number of people infected with HIV grew by 13 per cent.

After sub-Saharan Africa, south/southeast Asia has the highest number of people infected with HIV, according to UNAIDS, at 6 million. Latin America comes next, with 1.3 million people infected. The figure for North America is 860,000 people, and for western Europe 150,000 people. **C.M.**

Japan to merge science with education

[TOKYO] Plans to merge the Science and Technology Agency (STA) and the Ministry for Education, Science, Sports and Culture (Monbusho) into a new Ministry of Education, Science and Technology have been confirmed by Japan's administrative reform council, chaired by Prime Minister Ryutaro Hashimoto. This follows an interim proposal put forward by the council in August (see *Nature* 388, 815: 1997).

The decision emerged last week from a four-day meeting during which the reform council tried to finalize plans to cut the number of ministries and agencies from 22 to 13.

In contrast to lobbying from bureaucrats against the restructuring of other ministries and agencies, the plan to merge the STA and Monbusho has faced relatively little opposition. The restructuring is planned to take effect from 2001 and legislation will be submitted to Japan's parliament next year.

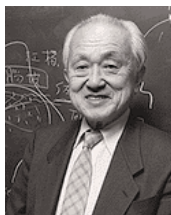
Monbusho officials are backing the merger, which they consider "crucial" to increasing the number of individuals in basic research and technology as Japan's population falls. The merger with STA, Monbusho says, is a "perfect opportunity to place more emphasis on science in higher education".

But research bodies funded through both Monbusho and STA are less positive. Many view STA's 'top-down' approach and Monbusho's education-oriented approach as fundamentally incompatible, and heads of government research organizations are watching nervously as the plans consolidate.

Minoru Oda, former director general of the Institute of Space and Astronomical Science (ISAS) under Monbusho, who also headed the Institute of Physical and Chemical Science (RIKEN) under the STA, is concerned the merger could jeopardize support for basic research, whose importance Monbusho has only recently begun to appreciate. "There is very little support and understanding for basic research in Japan," he says. "Many people, including both scientists and bureaucrats, have often missed the point that 'science' and 'technology' are separate issues."

One of Oda's main concerns is that ISAS might be merged with the National Space Development Agency (NASDA) under STA. ISAS, which formerly belonged to Tokyo University, is a successful academic institute carrying out basic research in space science using small low-budget satellites and rockets. NASDA, in contrast, is devoted mainly to the launch of large applications satellites and is staffed mainly by engineers.

Oda fears that a merger under the revamped ministry might appeal to politicians and the public, whose main concern is reducing the burden of bureaucracy. "The foundation for basic science in Japan is still



Oda: sees threat to basic research.

"might generate some potential benefits by breaking the bureaucratic wall between the two sides". But he adds the gap between their policies "may overshadow such benefits".

But supporters of the merger argue that it could give more flexibility to Monbusho's current funding system, which splits funds in a bureaucratic fashion criticized as rigid, inflexible and 'over-democratic'. Some propose converting national laboratories and semi-public institutes, such as RIKEN, to 'independent administrative agencies' to give them financial independence, as well as requiring greater accountability.

"The current funding system [under Monbusho] is unsuitable for basic research," says Keiichi Kodaira, head of the National Astronomical Observatory. "Turning research institutes into more autonomous 'agencies' might be worth considering, although this needs to be looked into more carefully," he adds.

very fragile; if such a merger goes ahead, support for basic science could well crumble and disappear," he says.

Akiyoshi Wada, emeritus professor of science at Tokyo University, admits the creation of an education and science ministry

The decision to merge STA and Monbusho was strongly promoted by Hashimoto, who was exasperated by the mishandling of nuclear accidents at STA's Power Reactor and Nuclear Fuel Corporation (PNC). He said in August he opposed an earlier proposal to upgrade STA into a ministry. STA officials say the agency probably would have become a full ministry of science but for the scandals surrounding PNC.

"With the creation of the basic law for science and technology last year, and an increase in budget for scientific research, STA's position was rapidly rising," says Fumio Saito, head of the administrative reform committee at the ruling Liberal Democratic Party. Saito lobbied against the merger. "Downgrading STA just because of what happened at PNC is frivolous," he says.

Part of the nuclear policy division within PNC, as well as other policy-making sections from STA, will now be incorporated into a strengthened Council of Science and Technology, Japan's leading science policy-making body within the Prime Minister's office.

The Environment Agency, which is to be upgraded into a 'ministry of environment safety', is to absorb the nuclear waste division of the ministry of health and welfare, as well as taking a stronger role in implementing policies on the emissions of greenhouse gases.

Asako Saegusa

Australia annuls Roche's native *Taq* patent

[LONDON] The legal tussle about rights to the thermostable enzyme *Taq* DNA polymerase has taken a new turn with a decision by the Australian Patent Office (APO) to withdraw patent rights to the enzyme in its native form issued to Swiss company Hoffmann-La Roche.

The decision has been welcomed by officials at New England Biolabs (NEB), which had led a challenge — together with the company Bresatec (now Bresagen) — to Roche's Australian patent on *Taq*, used extensively in laboratories as part of the polymerase chain reaction technique.

But Roche, which is also facing a court challenge to the US *Taq* patent from the laboratory equipment company Promega, has shrugged off the Australian decision. It argues that it has further information to back up its patent application which it has yet to present to the courts. (The company's patents to recombinant *Taq* and related products were upheld.)

The decision by the APO examiner focused on 'prior art', and in particular on claims by NEB and Promega, both of which have been selling versions of *Taq*, that its existence had been known to other

researchers before its claimed 'discovery' by the US company Cetus in the mid-1980s.

The examiner supported, for example, the claim that a thermostable enzyme extracted by Russian scientists in the 1970s was essentially identical to that described in the patent application. The rejection of this argument by the European Patent Office last year was largely responsible for Roche being awarded the European patent on all forms of *Taq* (see *Nature* 384, 100; 1996).

Donald G. Comb, president of NEB, claimed last week that the APO decision "affirms the view of many scientists in the world that this and similar patents to native *Taq* DNA polymerase should never have been issued". Officials from Promega express the hope that "the US court, as well as the EPO in upcoming opposition proceedings, will follow Australia's lead".

But Roche said on Monday (24 November) that it had "for formal reasons" not been able to present to the APO the "strong evidence" it had provided to the EPO refuting opponents' claims of 'prior art'. Roche says it is "confident" that it will win an appeal it has now lodged with the Federal Court in Sydney.

David Dickson

Misconduct office cuts backlog of alleged cases under review

[WASHINGTON] The US government office charged with investigating and ruling on alleged misconduct by government-funded scientists has cut its case backlog to a record low, says a report released last week. The Department of Health and Human Services' Office of Research Integrity (ORI) said that at the end of 1996, it had 48 misconduct cases formally under review and was studying allegations in 13 more.

"This is a tremendous improvement," ORI's annual report for 1996 says. It says that when the office began its work in 1992, it had a backlog of 70 cases and more than 600 unresolved allegations. In 1996, the office received 196 new allegations of misconduct, of which 62 were assessed in depth to decide whether an investigation should be launched. Of these, 80 per cent were resolved within the calendar year, with an average processing time of 29 days.

France plans reforms for *grandes écoles*

[PARIS] Claude Allègre, the French minister of national education, research and technology, is planning to reform the renowned *grandes écoles* to improve links with universities in research, teaching and student mobility.

Allègre has set up a commission to propose reforms, chaired by Jacques Attali, a former adviser to the late President François Mitterrand, and a product himself of the *grandes écoles*. The commission includes university and industry leaders, and leading researchers such as the geneticist Axel Kahn, and Georges Charpak, the 1992 Nobel prizewinner in physics.

Although the cream of students admitted to the *grandes écoles* generally enjoy much better facilities than their university counterparts, the universities have a stronger research base. Allègre has criticized the lack of a technology base in the *grandes écoles* as being partly responsible for what he claims is a lack of innovation among the elite who go on to run France's large enterprises.

FDA law aims to speed up drug approval

[WASHINGTON] After three years of contentious efforts to change the US Food and Drug Administration (FDA), congressional supporters saw their labours succeed last week when President Bill Clinton signed into law the FDA Modernization Act of 1997, the most significant change in FDA procedures in more than 30 years.

The law, which aims to speed the approval

of new drugs and medical devices, may also make access to experimental therapies easier for sick patients while FDA approvals are pending. To speed approval times, companies will be allowed to hire third parties to review mainly low-risk medical devices. Clinton said that the law means that the FDA "wins the gold medal for leading the way into the future". But critics say it will expose patients to risky drugs and devices that are given speedy approval for the financial gain of their makers.

'NIH should stipulate mentoring for women'

[WASHINGTON] The US National Institutes of Health (NIH) should require principal investigators (PIs) to foster the development of female scientists as a condition of receiving funding, a conference on NIH research on women's health recommended last week. "Mentoring should be a mandatory component of every funded PI award for an R01 grant," the draft recommendations from the conference state. "NIH would provide a stipend for the mentoring."

The three-day conference in Bethesda, Maryland, was sponsored by the NIH Office of Research on Women's Health. The mentoring recommendation was compiled by a working group on career issues. At present NIH does not require investigators on standard R01 grants to offer career support to female co-investigators.

Imperial College plans departmental merger

[LONDON] Imperial College of Science, Technology and Medicine in London is merging its departments of geology and Earth resources engineering and its Centre for Environmental Technology into a single teaching and research unit, to be known as the T. H. Huxley School of Environment, Earth Science and Engineering.

According to Sir Ronald Oxburgh, rector of Imperial, the school "will develop new areas of activity that were previously inhibited by the interdepartmental boundaries". The school will come into existence on 1 January, and Oxburgh intends to nominate John Beddington, currently head of the Centre for Environmental Technology, as its first director.

Russian minister rejects call to delay reforms

[MOSCOW] Russia's science minister Vladimir Bulgak has rejected a demand from trade unions to postpone for a year or so his plans for the reform of Russian science, which are likely to lead to wide job losses.

The unions had asked that the reforms, which are due to be presented to the cabinet

early next month, should be held back for further discussion. But Bulgak, who is a vice-prime minister, confirmed that the government intends to press ahead. Speaking at the House of Government in Moscow last week, he said a detailed reform programme would be drawn up at the beginning of next year, and the results made public in March.

Bulgak said that the aim of the reforms would be to adapt the country's scientific efforts to the current state of its economy. "Science is an evergreen tree, but it has some branches which are already dead and should be cut off," he said. Bulgak added that certain organizations were likely to lose the right to use the word "scientific" in their titles.

Biotech and genetics consultation planned

[LONDON] John Battle, Britain's minister for science, energy and industry, has confirmed plans to stage a nation-wide public consultation on the impact of recent advances in biotechnology and human genetics (see *Nature* 389, 222; 1997). "These developments offer hope," he said on Monday (24 November). "But they also raise difficult ethical questions, and there is a real fear that technological advances are outstripping our capacity to handle them."

Battle has promised that the government will host a number of "public events" next summer. No decisions have yet been taken on what form these will take, although possibilities are said to include 'consensus conferences' and 'deliberative polling' intended to measure shifts in attitudes among those provided with an opportunity to gather and discuss information on particular developments.

Synchrotron lab formally opened in Brazil

[SAO PAULO] Brazilian president Fernando Henrique Cardoso has officially inaugurated a long-anticipated federal laboratory, the National Synchrotron Light Laboratory. Initially planned more than a decade ago, the laboratory has been completed at a cost of US\$70 million.

The laboratory, whose first beamlines opened last summer, is described as "the largest civilian scientific project ever developed in Brazil". It was designed by Brazilian researchers, and houses a 1.37 GeV electron storage ring that acts as a radiation source for applications in materials research.

Correction

The photograph accompanying last week's story on Australian universities and described as being of Roderick West, the chair of a recent review of higher education, was in fact David Kemp, the new minister for employment, education and training. We apologize for the confusion.

Problems of nomenclature

Sir— You are to be commended for devoting a leading article¹ to the nomenclature problems affecting molecular biology. The text is, however, misleading in its implication that molecular biologists have no recourse to an official international committee that addresses matters of nomenclature.

The IUPAC-IUBMB Joint Commission of Biochemical Nomenclature (JCBN)² produces recommendations to aid communication of biochemical and molecular biological information and encourages scientists to use generally understood terminology. The committee is also responsible for *Enzyme Nomenclature*, the enzyme classification system used in several important electronic databases (see refs 3–5 for examples).

Problems of nomenclature are already seriously hampering biological research and information retrieval from public databases. If molecular biologists and biochemists are willing to tackle this important area, they should be reminded that JCBN has an established infrastructure to address nomenclature problems and is forming strong initiatives in the field of bioinformatics. The committee already has representatives from Swiss-Prot⁵ and the Nucleic Acid Database⁶ and is seeking to extend its interests because of the imminence of the problems faced.

The committee welcomes initiatives from groups in the research community to organize nomenclature within specialist areas, and is willing to advise on the design of nomenclature systems that conform to the conventions of IUPAC-IUBMB. All that is required is satisfactory financial backing, cooperation from the scientific community and a willingness to improve effective access to the large amount of data being generated.

Barry J. Whyte

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Sir— “What’s in a name? That which we call a rose/ By any other name would smell as sweet” (W. Shakespeare, *Romeo and Juliet*; 1594).

But if profligate nomenclature is used, cabbage and rose could become confused and the sweet smell of successful cloning could rapidly degenerate into the thorny issue of which name refers to which.

These are the types of issue with which we deal on a daily basis. We are trying, along with many in the field of molecular biology, to improve communication between scientists by approving a single unique symbol for each gene.

The HUGO/GDB Nomenclature team

headed by S.P. consists of classically educated scientists with a sound base in Latin, Greek and taxonomic classification as well as genetics. This enables us to achieve the goal of a unique, descriptive, memorable and consistent symbol and name for each gene locus.

It is true that one committee is not enough to cope with all the information, which is why there are nomenclature committees for the human (GDB), mouse (MGD), rat (Ratmap), *Drosophila* (Flybase) and yeast (SGD) to name but a few.

Within these designated bodies, particularly at the HUGO/GDB Nomenclature Committee, we encourage investigators in specific fields to set up their own committees, while giving them advice and encouraging the use of the nomenclature guidelines. A recent example is the Caspase Committee, which has assigned to the caspase genes a descriptive and systematic nomenclature. The committee is also now dealing with the variously named FLIP, Casper, FLAME, CASH and I-FLICE, so watch this space.

The majority of these gene nomenclature committees have evolved from organizations concerned with gene mapping, but are now attempting to broaden their perspective to serve a much wider community of biologists with interests in the organization, control, function and interrelationships of genes, not merely their position on the chromosome maps. Many molecular biologists have progressed from whimsy and promiscuity to evolve a systematic and methodical set of criteria for gene names and symbols.

However, until the large number of journals that publish in this field insist on the use of the approved symbols, communication will be severely hampered. High impact journals such as *Nature* have a special responsibility to ensure correct nomenclature is used, as they are

likely to be viewed as setting the standards.

The HUGO/GDB Nomenclature Committee is happy to deal with any queries about existing or new gene nomenclature and can be contacted at <http://www.gene.ucl.ac.uk/nomenclature/>.

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Sir— We are concerned by the profligate habit of assigning three-letter names to genes and their corresponding proteins. Apart from playing havoc with spell-checking programs, there is a disturbing tendency for such names to lack vowels, thus rendering their pronunciation inscrutable.

This problem is particularly common in our area of research, cell signalling. Is the protein tyrosine kinase *Lck*- lack, lock, luck, leck or even lick? Most distressingly, the natural pronunciation that springs to mind may be offensive in nature, or at the very least denigrating to the protein involved. Many of us struggle to reconcile euphony with civility when faced with the likes of *Crk*, *Drk* or *Suc*.

We implore researchers to take a more considerate and ethical approach in their gene and protein nomenclature to put an end to this acronym anarchy.

Lnc Puente

Srt Edmonds

Crp Arendt

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1. *Nature* **389**, 1 (1997).

2. <http://www.chem.qmw.ac.uk/iupac/jcfn>

3. EcoCyc <http://www.ai.sri.com/ecocyc/ecocyc.html>

4. KEGG <http://www.genome.ad.jp/kegg/kegg1.html>

5. Swiss-Prot <http://expasy.hcuge.ch/sprot/sprot-top.html>

6. Nucleic Acid Database <http://ndbserver.rutgers.edu/>

Not playing the game

Sir— *Nature* is known as a journal that publishes sound research papers based on accurate data. One would expect such accuracy also in data included in editorials.

The leading article “Spanish science is still at risk” (*Nature* **389**, 767; 1997) was wrong in saying that the budget of Spain’s scientific research council (CSIC) is lower than the fee paid for the transfer of a famous football player to an Italian club: the entire CSIC budget amounts to about

ten times that transfer fee.

With this budget, CSIC’s scientific production represents about 20 per cent of the Spanish total, and that in turn is 2.2 per cent of world science production. CSIC is one of the main research contractors with the European Commission in the current Framework programme.

César Nombela

(President)

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Art and science

Sir — In the art and science discussion¹ it is suggested that art's influence on science is thinner than the other way around. Although the yardstick with which we measure such influences is imperfect, the influence of art is not so thin in neurological science, the study of the mind in the brain.

The mind is expressed in fine art works by artists whose studios are natural laboratories for neurological science. Here are some examples:

- (i) The ability of artists to paint masterfully even after cortical damage has shown that talent is diffusely represented in the brain, unlike speech, language comprehension or memory, which have focal brain representations².
- (ii) Magritte's *The Rape* has led to insights about how faces are perceived in the two brain hemispheres in split-brain patients³.
- (iii) The selective emphasis on the left half of women's faces in portraits by Rembrandt, and by many other portrait painters over several centuries, has led to numerous psychological experiments, including discovering facial asymmetry in the manifestation of beauty in women's faces and speculating about

adaptive coevolution between face and brain⁴.

Because artistic productions reflect the mind in the brain, art holds many insights for behavioural brain researchers.

Dahlia W. Zaidel

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Sir — I should like to draw your attention to a little-known quotation from *The Notebooks of Raymond Chandler*⁵. On page 7 Chandler describes a "great thought" that occurred to him in February 1938:

"There are two kinds of truth: the truth that lights the way and the truth that warms the heart. The first of these is science, and the second is art. Neither is independent of the other or more important than the other. Without art science would be as useless as a pair of high forceps in the hands of a plumber. Without science art would become a crude mess of folklore and emotional quackery. The truth of art keeps science from becoming inhuman, and the truth of science keeps art from becoming ridiculous."

Charles Darwin realized that by solely doing science and by thinking only in theoretical terms his mind would change

into a machine, eventually depriving him of the possibility of experiencing happiness. As a consequence, Darwin resolved to read more poetry and listen more to music.

What Darwin experienced as an individual may well apply to the public and to science as a whole. People perceive science (without art) as a machine that continues to produce useful things but nevertheless detaches itself from human needs (and becomes inhuman). The growing public misunderstanding of science is not due to a lack of good explanations but to a lack of human aspects in its knowledge.

Getting artists and scientists to cooperate is crucial for future developments in both fields. Several years ago, a group of people in Germany founded a private university that no longer separates the arts and sciences but teaches both aspects of human endeavour as a unit.

Ernst Peter Fischer

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A different perspective

Sir — I quote from Martin Kemp's article (*Nature* **390**, 128; 1997) on *The Flagellation* by the artist Piero della Francesca: "Getting things right was integral to his contract with God's design of nature".

Unfortunately, *Nature* seems to have let its Designer down and to have broken the contract, by reproducing the picture the wrong way round.

P. B. Soul

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Saving British science

Sir — Yes, as you say in your leading article, science and engineering are centred on core disciplines (*Nature* **390**, 101; 1997), each giving a form of identity to the members of their communities and making distinctive contributions to the advance of knowledge and its applications — but now in ways more multidisciplinary than ever before.

To take your example of molecular biology, the headlines it captures rest essentially on continuing advances in knowledge and technical developments in other areas of research such as physics,

chemistry and engineering, and will continue to do so.

Each discipline should ensure that its role in contemporary science is understood and properly valued, but it must not lose sight of the essential unity of 'science'. That unity must be promoted in coherent cross-disciplinary advocacy. If we, in the 'broad church of science', forget that, how can we expect the Treasury accountants to understand? Divided, we fall.

The Royal Society, the Royal Academy of Engineering and the Royal Colleges of Medicine have the authority and the responsibility to speak for 'science', in the broad sense (it is a pity they are three, not one). Save British Science (SBS) also tries. A very coherent message was recently given to John Battle, the minister for science, by a group of 20 senior academics mustered by SBS; it included a chemical engineer, an astrophysicist and a clinician.

Unfortunately your article's emphasis on discipline-based advocacy can too easily turn into a narrow, negatively competitive struggle for a share of inadequate resources. Speaking for science as a whole may be less easy, but it is essential if the long-term health of science is to be preserved — and truer to the spirit of *Nature*.

J. H. Mulvey

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Soddy's economics undervalued

Sir — I was interested to read A. G. Maddock's review of Linda Merricks' book *The World Made New: Frederick Soddy, Science, Politics and Environment* (*Nature* **389**, 925; 1997).

I agree with the criticism that the book fails to give an account of Soddy's ideas on economics and monetary reform: Soddy gave up a distinguished career in research to elucidate and publicize, by both lecturing and writing, what he thought were the defects of the modern banking and monetary systems.

The New Europe Group and New Britain Movement: Collected Publications 1932–1957 (New Atlantic Foundation in association with the J. B. Priestley Library, University of Bradford; 1997) contains a section on "Professor Soddy: Lectures and Articles 1932–1957". These were all directed to a nonspecialist public and are very readable. Books by Soddy are held by the J. B. Priestley Library and are accessible for study on application to the librarian.

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Zen and the art of climate maintenance

Targets and timetables dominate the policy response to climate change. Is it wise to rely on a strategy that has yet to get off the ground? Or could Zen and social science suggest sensible alternatives?

**Steve Rayner and
Elizabeth L. Malone**

Zen Buddhism teaches that enlightenment comes not through scripture, ritual or good deeds but by breaking through mental boundaries imposed by established ways of looking at the world. So it may be a good omen that the United Nations climate convention is meeting in Kyoto next week, a historic centre of Zen teaching. The convention was adopted at the Rio de Janeiro Earth Summit in 1992. It concentrated on reducing greenhouse-gas emissions to preempt global warming. Since Rio, governments, delegates and nongovernmental observers have pored over the reports of the Intergovernmental Panel on Climate Change (IPCC), performed rituals of international negotiation and even attempted voluntary targets for emissions reduction. But despite the emergence of a scientific consensus about the reality of climate change, the world seems no closer to halting the rise in greenhouse-gas emissions and has barely begun to consider how it will cope with the consequences of not doing so. The time is ripe for a fresh look at climate policy strategy.

Policy proposals responding to the threat of climate change have almost exclusively focused on emissions reductions since 1988, when the goal of a cut of 20 per cent by 2005 was proposed at the Toronto Conference on Climate Change. This was based not on scientific assessment of what would be required to stabilize the atmosphere, but on the participants' assessment of what might be achievable politically. Unfortunately, even that now seems over-optimistic. In the decade since, the radiative forcing effect of greenhouse gases on the planet's surface has risen by a little more than half a watt per square metre, about a fifth of the total increase from the beginning of the industrial revolution to 1990. Concentrations of carbon dioxide have risen about five times the average for the decades between the industrial revolution and 1988. Of the member countries of the Organization for Economic Cooperation and Development that set themselves voluntary targets of a return to 1990 emission levels by 2000, only the United Kingdom and Germany have succeeded, but for reasons unconnected to climate policy. Meanwhile emissions from countries outside the organization seem set to double or triple over the next half century.

True, the journey of a thousand miles begins with a single step. But the record and prospects of achieving emissions reductions

suggest it would be prudent to expand the repertoire of climate-change policies, if only because our past emissions and the timetable for any plausible reduction programme mean climate change is already upon us.

Also the direct approach to mitigation through targets and timetables sidesteps other pressing issues of human welfare. Those most likely to suffer serious impacts from climate change are future generations of poor people in vulnerable parts of the tropics. Is incurring mitigation costs necessarily the best or most equitable way to help future victims if it leaves their parents and grandparents in a quagmire of poverty and resource degradation? Why are we so concerned about future generations of poor people in developing countries yet indifferent to those alive today? People's vulnerability to the impact of climate change depends on both the nature and extent of the impact and how they can respond. Might not decisive action to tackle destitution and resource impoverishment be a more effective allocation of global resources than force-marching the demise of fossil fuels? Such questions cannot even be posed because of the grip emissions reduction strategy has on policy.

Coping with unpredictability

Although climate change threatens poor people in vulnerable locations, it is unlikely to be the deciding factor in the welfare of human society as a whole. In fact, for much of the world's population, technological, economic, social and political change is likely to occur at such a rate that changes in the global climate of the order predicted by the IPCC will be barely noticeable. In such a rapidly changing world, it seems likely that long-term environmental issues will find it hard to compete for attention with immediate socioeconomic and political problems.

In creating scenarios, researchers often extrapolate from the present to posit a future that is more of the same. The future world of the IPCC assessment reports is essentially today's world, but more so: more people, more growth, and more technology (largely of the same sort). History suggests that such assumptions are unrealistic. An analyst in 1897 would be pressed to envisage even the broad outlines of the changes in technological capacity and distribution over the next 100 years. One nineteenth-century British parliamentarian said that, at the prevailing rate of emissions, London would be several feet deep in horse manure by the 1950s.

The rapid rate of socioeconomic and technical change relative to climate change

contrasts with the slower background rate of change of the natural world. Ecologists frequently warn us that it is not so much the amount of climate change that is dangerous but that the change will be too fast for ecosystems to adapt. On the other hand, society is changing faster. So the implications of the rate of climate change for society may be different from its implications for unmanaged ecosystems. Not only may societies adapt to climate impacts, but technological change may lead to more rapid displacement of fossil fuels than is conceivable today. Although there is no simple technological fix, there can be no fix without technological change. The problem is that there is no way of telling whether rapid social and technological change will prove a saving grace or another challenge to global environmental governance.

If decision-makers cannot predict the unpredictable, how can society face the prospect of profound change at an accelerating pace? The answer may be to focus on building responsive institutional arrangements that monitor change and maximize the flexibility of populations to respond creatively and constructively to it.

Approaching the climate issue from this starting point, that of assessing human vulnerability and social adaptation, may be far less amenable to concerted rational action by national governments than implementing emissions reduction targets. It also opens the way to discussion of the adaptive strategies that will be required, even to tackle the existing commitment to climate change through emissions. It also may be more relevant to stakeholders as it allows a varied response to local conditions. For instance, a measure designed to protect a coastal community from sea-level rise may have nothing in common with measures to stem desertification. Adaptation is a bottom-up strategy that starts with changes and pressures experienced in people's daily lives. This contrasts with the top-down approach of national targets for emissions reductions. The connections between emissions targets and people's

Might not decisive action to tackle destitution be a more effective reallocation of global resources than force-marching the demise of fossil fuels?

everyday behaviour and responsibilities seem less direct, even abstract. Designing adaptation strategies may be more sensitive to real trade-offs made by real people.

Adaptation strategies could also pave the way for effective emissions reductions with or without targets. Experience with adaptation could provide a complementary, perhaps an alternative, model for pursuing reductions based on how people in homes, factories and fields and on the roads can be empowered institutionally and technologically to change the way they do their business — whether or not there are formal targets and timetables.

In other words, policymakers should at least ask whether there is anything to be learned from adaptation to assist the process of actual emissions reductions (as distinct from the formal process of agreeing national targets). A forthcoming social science assessment, *Human Choice and Climate Change* (edited by S. R. and E. L. M., Battelle, January 1998), suggests that there might be. Such learning might take the form of developing social and technological strategies focused on institutional arrangements that routinely encourage the production of environmental goods rather than bads. In other words, decision-makers should encourage the generation of capabilities to exercise community responsibility and the dematerialization of industrial processes, without aiming at a particular goal for emissions reductions. The approach may seem as unpromising as the maze-like entrance to Kyoto's Kinkakuji temple (right), which artfully depresses visitors' expectations before suddenly confronting them with the breathtaking Golden Pavilion. Ultimately an indirect approach may yield higher rewards.

The assessment of research in *Human Choice and Climate Change* suggests that the view of climate change as an issue of social choices opens the range of actions within the commitment to emissions reductions. Instead of tracing a narrow line of causality from emissions to climate to impacts, we can explore how emissions-producing activities are embedded in social institutions and ways of life — and, then, what alternatives are possible within those institutional arrangements. Specifically, policymakers can:

- **Design policy instruments for real world conditions rather than try to make the world conform to a particular policy model.** A barriers-to-information model is the basis for much proposed climate-change policy. Underlying this is the assumption that there are well-behaved markets with traders who have perfect information; deviations from the ideal are barriers that can be removed by inserting the right information.

Unfortunately, even highly industrialized countries vary greatly from this model of information flows and barriers. In less industrialized countries, the variations are so

large as to render the model useless: many cannot carry out even the most elementary functions of government, let alone implement climate-change policies such as those addressing land and water use. In these conditions, the issue of optimizing across regulations, taxes, permits, education, and demonstration projects is academic. When development approaches treat fundamental structural differences as mere barriers, policy instruments cannot fail to fail.

The solution is to design information to fit the everyday perspectives of diverse stakeholders and to design policy instruments to suit specific conditions. Instead of trying to make the world conform to the rational choice model, we should attempt to understand how decisions really are made and shape information pertinent to decision-makers and their options.

- **Incorporate climate change concerns into other, more immediate issues such as employment, defence, economic development and public health.** Without a major policy stimulus (such as a carbon tax) or an unmistakable signal that climate change is real and threatening, any country is likely to delay the kinds of behavioural changes necessary to mitigate or adapt to climate change. Climate change will stay at the policy periphery, while attention will stay focused on core issues such as national economic policy or corporate manufacturing strategy.

The appropriate strategy is to build climate concern into the everyday concerns of people at the local level and the big concerns of policymakers at the national level. Joining climate-change issues to issues of social resilience opens the agenda to a broad range of focus areas, including economic development, institutional restructuring, provision of a variety of strategies, fostering civil society and strengthening indigenous arrangements (such as land tenure) that are working. Resilience encompasses not just preservation from harm (where possible) but also strengthening or establishing alternative economic activities and social structures.

- **Take a regional and local approach to climate policymaking and implementation.** In the daily lives of most people, local government is more salient than central government. Local government delivers or withholds services; it mediates between the citizen and the state through officials, such as police officers, who may have to monitor vehicle emissions, or building inspectors, responsible for seeing that new construction meets energy efficiency standards. Further-

more, more than 50 per cent of the world's population now lives in urban areas, where the density, mixture and physical layout of residential and commercial neighbourhoods all influence the energy intensity of the community. Many of these factors are more directly under the control of community governments than of national ministries.

However, almost all the climate-change policy research and analysis is aimed at high-level policymakers. Funding agencies tend to be national governments or organizations seeking to influence government policy or international negotiations. This is important, but not very helpful to a city manager, the manager of an aluminum smelter, the operator of a reservoir system, or a householder seeking guidance on how to do the right thing for the climate while doing the best for his or her citizens, stockholders and employees, consumers, or family members.

Policymakers can seek out and encourage local-level activities in many ways. For example, the Municipal Leaders Summit for Climate Change in New York in 1993 set up the Cities For Climate Protection programme. This was an extension of an earlier initiative linking 14 cities in the United States, Canada,



Europe and Turkey, designed to strengthen local commitment to reduce urban greenhouse-gas emissions, to research and develop best practices in pilot communities, to share planning tools and experiences, and to enhance municipal ties across national boundaries, especially between industrialized and less industrialized countries.

● **Direct resources to identify vulnerability and promote resilience, especially where impacts will be largest.** Whatever level decisions are made at, sustainability is about being nimble, not being right. Policymakers should balance the emphasis on linear goal-setting and implementation with attention to promoting social resilience through enhancing the ability to switch strategies as conditions change. This is particularly urgent where populations are vulnerable to the early impacts of climate change.

Central problems are to define vulnerability and resilience and to identify relevant indicators of each. Vulnerability includes risks to people, land and infrastructure—but just as important are political and economic systems and other institutional arrangements. Changes in regional patterns of habitability would exacerbate existing problems for the poor in environmentally vulnerable areas, such as low-lying tropical regions.

There is no standard framework for identifying sources of vulnerability, but clearly they are numerous and complex. Poverty is recognized as one of the most important correlates of vulnerability to hazard, but it is neither necessary nor sufficient for it. The very young and the old are often identified as especially vulnerable. Other categories invoked are differences in health, gender, ethnicity, education, and experience with the hazard in question. Empirical local-level studies reveal such complex mosaics of vulnerability as to cast doubt on attempts to describe patterns and trends at global or even regional scales. Vulnerability to global climate change is likely to be as complex.

The IPCC second assessment report has made a preliminary identification of regions and societies where climate-change impacts are likely to be most severe: for example, coastal zones and areas that are already warm and dry. However, social science warns us off broad pronouncements about relative vul-

nerability. Some researchers argue that the industrialized world is more vulnerable because of increasing interdependencies and rigidities in the industrial system and its supporting infrastructures. Other researchers argue that the vulnerability of the less industrialized world is greater as it depends on agriculture. The emphasis should be on collaborations wherever gains can be made in the capacities of societies to deal with problems that may result from and contribute to climate and other environmental changes. Building up both the social and the financial capital of the poor may be the best defence.

● **Use a pluralistic approach to decision-making.** The climate-change convention is an important symbol of worldwide concern about climate and about the persistent issues of global development that are bound up with it. But the real business of responding to climate concerns may well be through smaller, often less formal, agreements among states; states and companies; companies and nongovernmental organizations and communities. The process is likely to be messy and contested, but it should make the most of diversity and the variety of decision strategies available.

At the international level, governments are keeping a tight grip on their prerogatives to represent national interests in climate negotiations, while domestically the interests that are represented as national are shaped by the interaction of policy networks including bureaucracies, business and citizen groups. Although the institutional structure is biased in favour of some groups, institutionalized patterns of domination are far from immutable. Moreover, the process through which choices articulate across scales from the local to the global is not a linear mechanism that produces the most rational alternative at the next higher or lower scale. Rather, the connections may resemble the interconnecting staircases in an M. C. Escher woodcut; actions at one level may directly impact decisions made several levels away and the direction of causation may not always be clear. Through these connections, even the weakest and most disenfranchised can find the means to influence the activities of the strong.

What will connect the diverse elements of a plural policy approach? The goal of creating resilience provides the theme, with resilience defined by a society's capacity to draw on different ways of using resources and distributing goods and to switch from strategies that are not working to ones that will. Each society needs to have within it many working methods of resource management. A society that uses one strategy only will be extremely vulnerable to change. Complex, overlapping, plural, interdependent civic institutions embodying diverse combinations of several basic strategies extend capabilities to develop in a sustain-

able fashion, even — especially — when confronted with surprise. Strategy switching within these systems can be very rapid.

Policymaking that links the local and global levels requires extension of civic life, both as civic science (linking scientific and technical knowledge with local knowledge and craft skills) and civic society (associational links outside governments and markets) at all levels to complement the market and government. Similarly extending integrated assessment analysis and enquiry will enable scientific efforts to provide information at all levels — not only global and national, but companies, nongovernmental organizations and households.

The first essential for policy in a complex world is to resist the urge to declare one viewpoint true and to reject others. This is neither mindless relativism that says one idea is just as good as any other nor a recipe for passivity and abdication of choice. Where people argue either about the way the world (natural and social) actually works or about the way the world ought to work, we are likely to find ourselves facing competing partial truths. To commit oneself, family, company, community or country to just one of these viewpoints is to gamble that it will turn out to be right and the others wrong. It is far more likely that all will be partly right and partly wrong. Recognizing this and stewarding the institutional pluralism for maintaining different viewpoints and a rich repertoire of policy strategies is what promoting social resilience, sustainable development and climate-change governance is all about.

The focus on targets and timetables for emissions reduction simplifies and categorizes climate change as a distinct problem. In so doing it domesticates a large, complex and unruly set of life's circumstances as capable of solution through rational analysis, goal setting and policy implementation by technocratic élites. But targets and timetables essentially represent the end-of-pipe or pollutant-by-pollutant approach to environmental management on a global scale. This approach is increasingly recognized as obsolete at local and national levels. Rather than applying lessons from local experience to global environmental change ("think locally, act globally"), policymakers appear intent on repeating the experience. Questioning the policy emphasis on targets and timetables calls into question not only whether the emphasis is right, but also whether an exclusively rational technocratic approach to policymaking is appropriate at all.

The time is ripe for a Zen-style rethinking of approaches to climate policy that breaks through the mental boundaries that today define our understanding of policy options. Will Kyoto be the place to do it?

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The time is ripe for a Zen style rethinking of approaches to climate policy that breaks through the mental boundaries that define our understanding of policy options.

A cancer therapy resistant to resistance

Robert S. Kerbel

The relentless growth of tumours depends on a good blood supply, and thus on subverting host mechanisms for making blood vessels. Attack on that process provides a promising route for treating cancer, but one that may take some years to reach clinical fruition.

Dozens of drugs are used to treat cancer and even more are currently in preclinical or early clinical development. Unfortunately, many factors conspire to limit their effectiveness, including problems of delivery and penetration¹, and a modest degree of selectivity for the very cells they were designed to eradicate. But probably the most important — and certainly the most frustrating — of these limiting factors is drug resistance. Some cancers, such as prostate tumours and melanomas, are intrinsically resistant to most anti-tumour drugs. Others, ovarian carcinoma or small-cell lung cancer for example, respond to chemotherapy and often disappear altogether, only to return later as drug-resistant

tumours. This is acquired drug resistance, and it is encountered in about 30 per cent of all cancer patients undergoing chemotherapy².

Tumour cells are a rapidly moving target because of the instability and consequent plasticity of their genomes. Events such as partial or complete deletion of chromosomes, amplification of genes, translocations or rearrangements of chromosomes, and simple mutations ensure efficient selection and overgrowth of drug-resistant tumour cells during and after chemotherapy. But there is a characteristic of tumours that may turn out to be the Achilles heel of acquired drug resistance. This is their strict dependence on recruiting new blood vessels

(angiogenesis) from the host, which they need for the delivery of nutrients and growth factors that enable them to grow unchecked³ (see box, overleaf).

On page 404 of this issue⁴, Boehm *et al.* report that chronic, intermittent therapy of three different mouse tumours with an inhibitor of angiogenesis called endostatin⁵ did not lead to any traces of acquired resistance (Fig. 1). In one set of experiments, mice with a subcutaneous lung tumour were subjected to six successive cycles of endostatin treatment. For each cycle, the tumour was allowed to grow (or regrow) until it reached about 3 per cent of the body weight of the mouse. Endostatin was then infused daily until the tumours regressed and were no longer palpable. The response was always the same — palpable tumours disappeared even after the sixth successive round of endostatin therapy (initiated about 140 days after the start of treatment).

In contrast, standard chemotherapy, using maximum doses of the drug cyclophosphamide, resulted in partial resistance by the third cycle of treatment, and complete resistance (that is, no response) by the fourth cycle. Moreover, two other mouse tumours (a melanoma and a fibrosarcoma) also failed to develop any resistance to repeated cycles of endostatin therapy. Perhaps the most significant finding was that all treated tumours eventually became indefinitely dormant even when endostatin treatment was halted. This startling event, the basis of which is unknown, occurred after a variable number of therapy cycles (two to six), the number of which depended on the particular tumour being treated.

The idea of using inhibitors of angiogenesis to avoid acquired drug resistance⁶ was based on the hypothesis that endothelial cells with a normal complement of chromosomes, given their relative genetic stability, should be far less prone to developing mechanisms of resistance than tumour cells (see box); endothelial cells are the progenitors of new blood capillaries. It is interesting that although a patient's tumour usually becomes progressively resistant to the toxic effects of chemotherapy, normal, drug-sensitive dividing cells in the same patient do not. For example, the degree of bone marrow suppression seen in patients with breast cancer or small-cell lung cancer remains the same, even after six cycles of combination chemotherapy^{7,8}.

A rigorous test of this hypothesis has had to await the arrival of two powerful agents that directly inhibit the formation of new blood vessels — angiostatin⁹ and endostatin⁵. These two drugs are superior to other angiogenesis inhibitors, which are either less potent or only inhibit angiogenesis indirectly¹⁰. For example, targeting vascular endothelial growth factor, a strong stimulator of angiogenesis, is likely to result in the

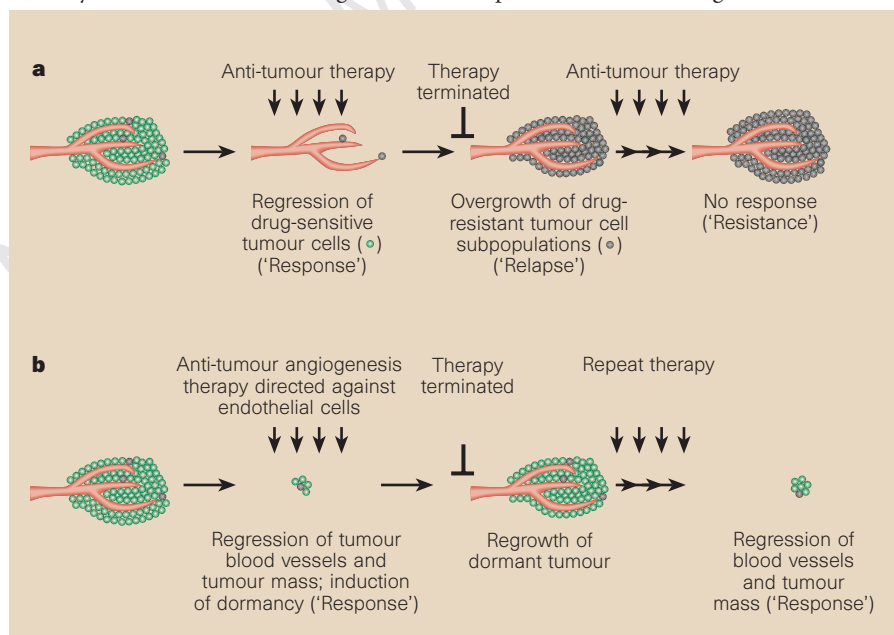


Figure 1 Anti-tumour drugs induce drug resistance in many tumours whereas angiogenesis inhibitors do not. **a**, Successive cycles of therapy using anti-tumour agents such as cyclophosphamide, which directly target genetically unstable tumour cells, leads to acquired drug resistance as a result of selection of drug-resistant tumour cells. **b**, In contrast, as shown by Boehm *et al.*⁴, repeated treatment with angiogenesis inhibitors such as endostatin, which directly target the normal (and genetically stable) endothelial cells of the tumour's developing blood vessels, does not induce resistance in tumour cells or host endothelial cells. By attacking the endothelial cells of the new blood vessels, angiogenesis inhibitors destroy the source of nutrients for the growing tumour, which regresses and, in the case of treatment with endostatin, becomes dormant.

emergence of tumour-cell variants that will be able to induce angiogenesis by secreting alternative growth factors^{11,12}.

The results of Boehm *et al.*⁴ are unprecedented and could herald a new era of cancer treatment. But that era could be years away. For example, the angiogenesis inhibitor used in their experiments (endostatin), although more potent than any other such inhibitor known, is a protein and as such has to be injected subcutaneously or intraperitoneally. Given the probable chronic nature of anti-angiogenesis therapy, oral administration of such drugs will be necessary to ensure good patient compliance. There is also the matter of the cost of using biologically based drugs for such a therapy (they are very expensive), and the prospect of toxic long-term side effects. In short, what may be needed is an anti-angiogenesis drug equivalent of aspirin. Finally, long experience tells us to be cautious in equating the results of experiments using tumours in mice to spontaneous tumours in humans¹³.

Nonetheless, we may now have a strategy that has a chance of defeating what has always seemed an inevitable and invincible property of malignant tumours. Given that various anti-angiogenesis drugs have recently entered clinical trials in cancer patients, it should not be too long before we see whether they are able to resist inducing drug resistance in humans as well as mice. There could just be some light at the end of the blood vessel after all. □

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Tumour growth and blood-vessel supply

The concept of treating tumours by inhibiting their ability to recruit new blood vessels (angiogenesis) has been around for more than 25 years¹⁴. It is based on several key principles.

● Tumour growth.

Solid tumours cannot grow beyond microscopic sizes without forming new blood vessels, and will remain dormant, or even regress, if angiogenesis is prevented or is not induced.

● Blood-vessel formation.

Tumours themselves are partly responsible for switching on blood-vessel formation, which they achieve by producing stimulators of angiogenesis and lowering their production of inhibitors of the

process¹⁵. Both genetic and environmental events (such as lack of oxygen) appear to contribute to the switching on of new blood-vessel growth. The former include genetic mutations known to be critical to tumour progression, such as activation of the *ras* oncogene and inactivation of the *p53* tumour-suppressor gene^{16,17}.

● Endothelial cells.

These cells line the insides of new blood vessels and express many different molecules compared with those in mature blood vessels. Such differences can be exploited for drug development. For example, the endothelial cells of new blood vessels have receptors

that bind to vascular endothelial growth factor, a potent inducer of (tumour) angiogenesis. These receptors make the endothelial cells of newly formed blood vessels selectively sensitive to agents such as antibodies directed against vascular endothelial growth factor or the receptors themselves^{18,19}. Moreover, because vascular endothelial growth factor enables endothelial cells to survive, agents directed against it result in regression of newly formed immature vessels^{20,21}, which in turn results in tumour regression. The same may be true for certain other inhibitors of angiogenesis, such as endostatin.

R.S.K.

Polymer synthesis

For the living there is hope

David A. Tirrell

On page 386 of this issue¹, T. J. Deming reports a new method of synthesizing amino-acid-based polymers — a 'living' polymerization that should yield materials with precisely controlled structures, and with unprecedented combinations of physical properties.

It has been 50 years since Woodward and Schramm published their brief paper² *Synthesis of Protein Analogs*, in which they described the ring-opening polymerization of *N*-carboxy- α -amino-acid anhydrides (NCAs) to produce polypeptides of high molecular weight (Fig. 1). The polymerization was initiated by the fortuitous presence of water, and although the products could be cast into "tough, clear, mechanically stable films", precise control — even accurate determination — of the molecular weights and molecular-weight distributions of these 'protein analogues' was not possible. Despite decades of vigorous investigation³ of the polymerization of NCAs, the situation has changed little, and the products of NCA polymerization are still poorly understood.

But now Deming has polymerized NCAs by zero-valent nickel catalysts under 'living' polymerization conditions; that is, conditions free of termination and chain transfer¹

(Fig. 1). Living polymerizations allow the synthesis of polymers of predetermined molecular weights and narrow molecular-weight distribution; and, perhaps more importantly, the preparation of well-defined block copolymers in which long sequences of each of the individual monomer residues are linked together at a single site⁴. Deming shows that these advantages of living polymerizations, which once were reserved for a small subset of polymerizable monomers, can now be extended to NCAs and to the preparation of high-molecular-weight polypeptides and block copolypeptides with unusual and useful properties.

Block copolymers have played an important part in materials science and technology because they allow the effective combination of disparate properties in a single material. Block copolymers of styrene and dienes, for example, are rubbery at room temperature (a characteristic of the polydiene phase) but can be moulded at temperatures above the glass transition of the polystyrene phase.

This distinguishes the block copolymers from most conventional rubbers, which must be chemically cross-linked (vulcanized) in order to withstand the stresses that they encounter in use. Because chemical

cross-linking is irreversible, it must be done while making the final part; and cross-linked rubber is difficult to reprocess. The 'cross-linking' step for styrene–diene block copolymers is instead a physical association of chains in the glassy polystyrene domains: the tendency of the two different chain sections to clump together, like with like. This association is robust enough to bear loads at room temperature, but is readily reversible upon heating.

Quite apart from their technological uses, block copolymers raise questions about the physics of macromolecular assembly and phase behaviour. How does the chain reconcile the wishes of the blocks to avoid one another when they are inextricably tied together?

Well-defined block copolymers assemble spontaneously into a variety of intriguing nanostructures, and other, aligned nanostructure arrays can be made using fluid flow or other fields⁵. Because of this, block copolymers have enjoyed great commercial success, as well as the ardent attentions of polymer physicists.

But block copolymers of amino acids have been little studied, largely because our synthetic methods do not have fine enough control to produce well-defined structures⁶. The same is true of the synthesis of block copolypeptides for use as biomaterials or as selective membranes—the potential advantages of the protein-like architectures have remained unrealized for want of adequate synthetic tools.

The method introduced by Deming¹ promises to change that. Deming treats the monomer of interest with the zero-valent nickel complex $\text{bipyNi}(\text{COD})$, where bipy is 2,2'-bipyridyl and COD is 1,5-cyclooctadi-

ene. Adding the NCA yields an initiator molecule that can be isolated and that remains active towards further ring-opening polymerization, and the target polypeptide can be prepared with essentially 100 per cent yield.

Claims of new 'living' polymerization methods must be evaluated with care. The ideal living polymerization is characterized by fast initiation and an absence of the termination and chain transfer steps that in most polymerization systems compete with propagation of the growing chain (Fig. 1). If these conditions are realized, all polymer chains begin growing at about the same time and continue to grow until the monomer has been exhausted. The average number of monomer residues per chain is then simply the molar ratio of monomer to initiator, and the distribution of chain lengths is described by Poisson statistics⁷.

Deming shows that these conditions have been met in the polymerization of NCAs by $\text{bipyNi}(\text{COD})$. The distribution of chain lengths is narrow, consistent with Poisson statistics, and the rate of polymerization is proportional to monomer concentration, indicating that the number of active chain ends remains constant throughout the reaction.

It is the absence of termination and transfer that makes living polymerization so powerful for synthesizing block copolymers. Because the growing chains remain active even after the monomer has been exhausted, adding a second monomer at that stage results in the growth of a second block distinct in composition from the first. Proper choice of monomers allows one to engineer the kinds of combinations of properties described above: rubbery and glassy; hydrophilic and hydrophobic; conducting and

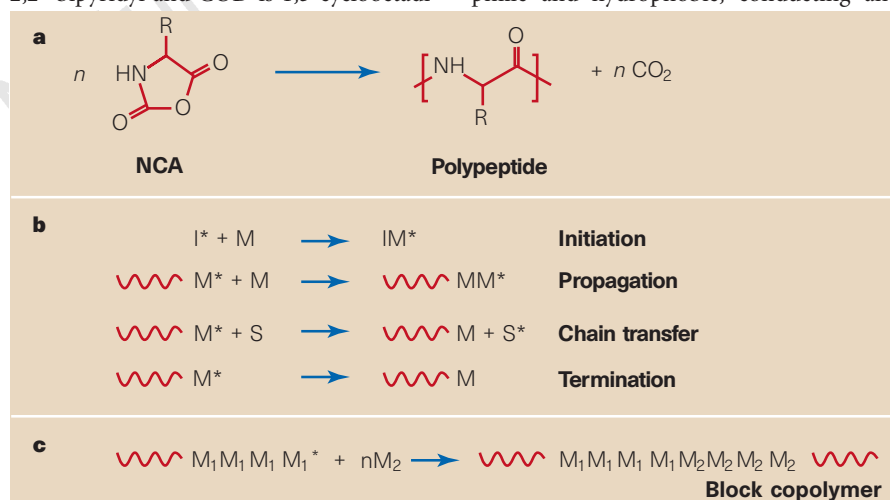


Figure 1 Synthesis of polypeptides by ring-opening polymerization of *N*-carboxy- α -amino-acid anhydrides (NCAs). **a**, Reaction of n moles of the NCA produces one or more moles of polypeptide and n moles of CO_2 . **b**, Chain-growth polymerizations involve initiation, propagation, chain-transfer and termination steps. Here the asterisk is an active species (derived from initiator (I) or monomer (M)) capable of further monomer addition, and S is a solvent or other species that ends the growth of one molecular chain and starts the growth of another. In living polymerizations, the termination and transfer steps are absent. **c**, Block copolymers are formed by the polymerization of the first monomer (M_1) to exhaustion, followed by the addition of a second monomer (M_2).



100 YEARS AGO

In your account of the late Dr. Haughton ... I see no mention of a somewhat fantastic instance of his versatility – namely, his investigation into the most merciful way of hanging criminals. It was, I believe, entirely owing to him that the present method of the "long drop" was introduced. According to the older method the rope was so arranged that the culprit fell barely knee deep, all the rest of his body being in view above the scaffold. He died usually by strangulation, sometimes combined with apoplexy, after what seemed to be a protracted agony. Now, he is allowed to fall through some 10 feet, more or less, according to his estimated bulk and weight, and he dies with a broken neck more painlessly than virtuous persons in their own beds. The problem was to find out the length of drop that would suffice to break the neck bone, but would be insufficient to tear off the head. Dr. Haughton experimented on the tensile strengths of the spine and of the muscles, and he published a formula for the length of drop, dependent on the height and weight of the culprit. ... It should be mentioned that a case actually occurred in which the drop was too deep, and the head of the criminal became wholly detached, and the legal doubt arose whether under those circumstances the sentence of being "hanged by the neck" had been duly carried out. From *Nature* 25 November 1897.

50 YEARS AGO

In *The Condor* of July–August 1947 (49, No. 4), Dean Amadon gives an account of an investigation which he has made into the weight of the largest known bird. The ostrich (*Struthio camelus*), largest of living birds, was far surpassed in size by the elephant bird (*Aepyornis maximus*) of Madagascar; the larger of the New Zealand moas such as *Dinornis* were intermediate in size. The moas and elephant birds were exterminated by the natives of these islands and are known only from sub-fossil remains of skeletons, eggs and feathers. From *Nature* 29 November 1947.

Many more abstracts like these can be found in *A Bedside Nature: Genius and Eccentricity in Science, 1869–1953*, a 266-page book edited by Walter Gratzer. Contact David Plant. e-mail: subscriptions@nature.com

insulating; and so on. A living polymerization method for NCAs should lead to block copolypeptides with new and useful properties.

Deming has demonstrated successful synthesis of such materials, and has created a new family of polypeptides that link combinations of acidic, basic and hydrophobic domains, all with excellent control of molecular architecture. The prospects for application in biomedical engineering, drug delivery and selective separations appear to be excellent. When it comes to polymerization, "For the living there is hope, but for the dead there is none"⁸. □

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Homing in on vertebrates

Joseph L. Kirschvink

Imagine being set adrift in a canoe in the middle of an ocean. Which way would you paddle? Most humans would be as lost as lost can be, but creatures such as pigeons, turtles and whales have no difficulty navigating in such circumstances. How they do so remains one of the biggest mysteries in the behavioural sciences, at the centre of which is the question of how organisms might sense the Earth's magnetic field and use it for navigation and homing (a topic with a chequered history — see box, overleaf). The mystery, however, is gradually being solved, and the latest instalment in the story comes in Walker and colleagues' study of rainbow trout (page 371 of this issue¹).

All known sensory systems have specialized receptor cells designed to respond to the external stimulus, and these are always coupled to neurons to bring this information to the brain. In modern times the main

objection to claims that magnetoreception is genuine was biophysical² — that there was no evidence of appropriate receptors.

The first clue towards meeting this objection came in an obscure place, the teeth of small molluscs. The late Heinz A. Lowenstam discovered that the major lateral teeth of the chitons are capped by massive amounts of a hardening agent, the permanently magnetic mineral magnetite (Fe_3O_4) (Fig. 1). Later, with the development of ultrasensitive superconducting moment magnetometers, came the recognition that tiny (parts per billion) levels of biogenic magnetite are naturally present in animals as diverse as insects, birds, fish and even humans^{3,4}. The final crack in the biophysical arguments against magnetoreception came with the discovery of the magnetotactic bacteria and protists (Fig. 2), which possess linear chains of either single-domain magnetite or greigite (Fe_3S_4). They provide unequivocal examples of biological activity being influenced by the geomagnetic field.

Magnetite seems made for magnetoreception. A typical magnetotactic bacterium is only a few micrometres in size, but contains enough magnetite to make its rotational energy in the geomagnetic field exceed the thermal background energy by factors of 20 or more. The cells are very good, passive compasses, and will align with the Earth's magnetic field even when dead. The equivalent of only a single magnetotactic bacterium, connected to a single sensory neuron in a higher animal, could give that animal — ant, honeybee, trout or even whale — an extraordinarily good magnetic compass sense.

If this were all of the magnetite used for magnetoreception, finding and characterizing the receptor would be a needle-in-the-haystack operation. Fortunately, things are more complex. There are at least two types of response to the geomagnetic field — a simple compass, and another which is the result of

sensing small fluctuations in the intensity of the background field. This latter sense has been implicated as a component of the navigational 'map' used by whales, turtles and birds⁵. Extensions of the biophysical analyses indicate that an array of a few thousand to a million magnetite-containing cells could yield responses to total intensity fluctuations of better than 0.1 per cent, as is observed behaviourally⁵, and that this entire receptor system could fit within a 1-mm cube and yet have a magnetite content of no more than 1 part per million (ref. 3). There is still no requirement for the receptors to be concentrated into such a small volume, but these calculations make the odds of finding them much better than previously thought.

Simple experiments using short but strong magnetic pulses (which exceed the coercive force of the magnetite) have shown that both the magnetic compass and intensity sensory systems involve the use of permanently magnetic materials such as magnetite^{6,7}. Linear chains of single-domain biological magnetite crystals suitable for magnetoreception are easy to extract from animals and image⁸ (Fig. 3); and electrophysiological studies in birds have consistently identified fibres in the ophthalmic branch of the trigeminal nerve as the carriers of magnetic-field information⁷.

So to Walker *et al.*¹, who have made two truly important advances. First, they have developed a simple laboratory conditioning regime for training rainbow trout to respond to magnetic cues. In principle, this could be extended to other vertebrates to tackle such

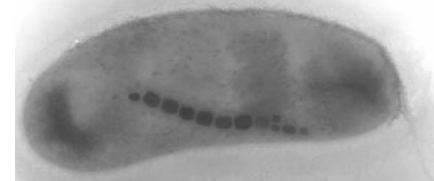


Figure 2 A freshwater magnetotactic bacterium. The chain of dark objects is composed of crystals of magnetite, which have the proper size and shape to behave as perfect, single magnetic domains. The largest crystals are about 70 nm in length. (Photo courtesy of A. Kobayashi.)

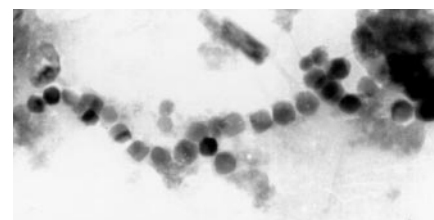


Figure 3 A linear chain of biogenic magnetite crystals, extracted from tissues in the frontal region of the sockeye salmon⁸, *Oncorhynchus nerka*, a close relative of the rainbow trout, *O. mykiss*. These are also single magnetic domains, with crystal alignments similar to those in magnetotactic bacteria. (Photo courtesy of S. Mann.)



Figure 1 Magnetite-bearing teeth of the mollusc *Cryptochiton stelleri*. Each tooth is about 1 mm in size, and capped with a layer of black magnetite (Fe_3O_4). (Photo, H. A. Lowenstam.)

From Mesmer to animal magnetism

Over the years, many examples of sensory systems that humans apparently do not possess have popped up in interesting places among the vertebrates. Some are extensions of known senses – echolocation in bats and whales, infrasound and ultrasound detection in birds, and the sex-pheromone receptors (the vomeronasal system) in the nose of most higher vertebrates. Others, such as the electroreceptive organs of sharks and rays, and the infrared detectors of snakes, depend on highly specialized receptor cells. These discoveries generated little scientific controversy.

Not so biomagnetism, which in the late eighteenth century received an early bad rap from the activities of Franz Anton Mesmer and his followers. The mesmerites claimed that they could cure disease

by exposing patients to magnetized objects, a claim debunked by a commission (which included Benjamin Franklin) appointed by King Louis XVI. The subject then fell into a long period of disrepute until the 1940s, when experiments suggesting that pigeons might use geomagnetic cues during homing generated great interest. These, however, were difficult to reproduce; likewise, conditioning experiments designed to elicit magnetoreception in the laboratory also failed.

The main stumbling block was in seeing how a geomagnetic stimulus could be converted into a signal that an individual cell could detect. Back-of-the-envelope calculations ruled out most of the obvious methods (paramagnetism, electrical induction, the Hall effect, nuclear magnetic resonance). The simplest

strategy – that of having a small permanent magnet – was dismissed on the grounds that there were no physiological ferromagnetic materials². As discussed in the main text, the discovery of magnetite eventually knocked that argument on the head.

Meantime, behavioural experiments kept on coming up with apparent geomagnetic effects on animal behaviour. But it took nearly two decades to realize that the geomagnetic compass used by adult birds was programmed to be ignored if other orientation cues (such as a Sun or star compass, polarized skylight, infrasound and ultrasound) were present. These orientation cues constitute a complex but consistent web of interacting responses, which are used not only by birds but in all major vertebrate groups and many invertebrates (reviewed in ref. 5). **J.L.K.**

electron microscopy and electron diffraction are required to confirm that the iron oxide is indeed magnetite.

A huge range of organisms can sense magnetic fields⁵. Do humans remain an exception? We certainly have a trigeminal nerve, with an ophthalmic branch, and we can also make biogenic magnetite. At least one other vertebrate sensory system thought to have been lost in the final stages of human evolution – the sex-pheromone-sensing vomeronasal organ – has recently been found to be both present and functional⁹ (human vomeronasalins now form a booming perfume industry). Other effects, such as the ability of a 1-millitesla static field to elicit epileptiform activity in patients preparing for brain surgery¹⁰, have also emerged. Finally, some humans, particularly Polynesian navigators, seem able to judge direction in the absence of all obvious cues (Sun, Moon, stars, waves and so on)¹¹. So there is hope for our lost canoeist – the final word on the existence of human magnetoreception has certainly not yet been written. □

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questions as threshold sensitivities and frequency response, as has been done with similar conditioning experiments with honeybees⁶.

Second, and even more excitingly, they have traced the sensory nerves back to possible magnetoreceptor cells. After confirming that the ophthalmic branch of the trigeminal nerve in fish contains magnetically receptive fibres, as it does in birds⁷, they used a lipid-tracing dye to map the fibres back to the brain and to the location of putative receptor cells. Attempts to trace the avian magnetoreceptive nerves have failed because of the problem of identifying magnetite crystals in optical sections. Walker and colleagues' application of confocal laser microscopy provided the techniques for both tracing individual neurons back through a complex three-dimensional path and, by calibrating the confocal reflections with magnetotactic bacteria, for identifying possible magnetite crystals in the target cells.

Note, however, that the iron oxide mineral has not yet been identified conclusively. The cells containing the confocal reflections have distinctive shapes and always lie within a discrete sublayer of the olfactory lamellae

(at the tips, near the distal terminals of fine branches of the trigeminal nerve), and the particles have similar size and shape to those extracted from salmon⁸ (Fig. 3). But follow-up studies with conventional transmission

Extraterrestrial impacts

The big splash

Jan Smit

Seventy per cent of the Earth is covered with sea water, and most of that is ocean, so most of the large asteroids and comets striking the planet should hit deep water. But very little remains of these impacts – virtually all evidence of large impacts, in the form of craters and associated debris, is found on land. On page 357 of this issue¹, Gersonde *et al.* describe the evidence for and consequences of the impact of an asteroid, 1–4 km in diameter, in a 5,000-m-deep ocean basin, 1,500 km southwest of Chile. The asteroid hit the Earth in the late Pliocene, some 2.15 million years ago.

The new results come from three piston cores, taken in the course of the 1995 FS

Polarstern expedition to the Bellingshausen Sea in the Southern Ocean. This expedition was dedicated to finding more remains of this, the only known oceanic impact, because piston cores, taken 30 years ago in the same area by the USNV *Eltanin*, yielded the iridium anomaly typically associated with extraterrestrial impacts, and melted and unmelted meteoritic debris. This debris identified the impactor as an asteroid, a basaltic achondrite which was named the *Eltanin* meteorite. But many questions remained.

The *Polarstern* cores were taken in different depths of water – one on a seamount (depth 2,707 m), one at intermediate depth

(3,965 m) and one on the Bellingshausen abyssal plain (4,961 m). All three yielded a complete sedimentary sequence across the impact 'horizon', although only the core at intermediate depth penetrated through the entire sequence. The cores all reveal a similar sequence, one that is consistent with an oceanic impact and bears a strong resemblance to the sequences found in the Gulf of Mexico that were deposited as a result of the Chicxulub impact on the Yucatán, Mexico, 65 million years ago.

A crater has not been found, however, and together with the absence of silicate debris on the ocean floor, that implies that the impact never penetrated into the basaltic basement of the ocean floor. From work with early impact models it seemed that a small (< 1-km) asteroid could penetrate to the sea floor and leave a crater, but newer models² support the conclusion that the Eltanin bolide may have been as large as 4 km without actually striking the sea floor. This is probably part of the reason that craters have thus far not been found on the deep ocean floor.

Nonetheless, the new cores show that the impact must have been substantial and disturbed the sea floor over hundreds of kilometres. Extremely strong currents resulting from the opening and closing of the transient 'oceanic' crater removed large amounts of sediments as 'rip-up clasts' and deposited them elsewhere. Gersonde *et al.* speculate that some sediments were probably blown away from the sea floor and were globally dispersed as ballistic ejecta. It seems that these may have landed on the Transantarctic Mountains, providing a nice third alternative explanation for the origin of some puzzling diatom-bearing deposits that were earlier explained as wind-blown dust or glacial till deposits³.

The *Polarstern* core sequences show a threefold subdivision of sedimentary units wedged between packages of undisturbed

sediments (see Fig. 3 on page 359). The lowermost unit consists of large blocks and slumped material that was swept along by the violent currents initiated by the impact. Because the cores were taken at different depths, it is possible to identify the blocks' origins. On the seamount, lumps of clay originating from the abyssal plain below the carbonate compensation depth (below which almost all carbonate shells dissolve, and only carbonate-free clays are deposited) were found well above that depth. Conversely, blocks of calcareous sediments from the seamount were swept into the abyssal basin. This lowermost unit has been traced in surveys over a wide area as a seismically transparent unit, meaning that it is not a layered but an acoustically uniform sediment. It fills topographic lows to a depth of up to 60 m and extends over hundreds of kilometres.

The overlying second unit consists of a laminated, grain-size graded sand. The laminae indicate deposition by currents, although not as strong as those involved in the basal unit; the grading indicates settling from suspension. Apparently, the ocean was still in motion at this stage, as a consequence of the impact, but currents were not strong enough to erode sediments away or prevent sedimentation of smaller particles. The top of this second unit contains the first, coarse, meteoritic debris (Fig. 1), which landed on the sea floor after a journey through space, the atmosphere and the water column, taking a minimum of four hours.

The third uppermost unit represents the slow deposition of fine, suspended material. Residual currents were then too weak to move particles over the sea floor, except in the layered basal part of the unit which is strongly enriched in meteoritic debris. The settling of such fine material may have lasted for weeks, but the process was still fast enough to prevent colonization of the sea floor by burrowing organisms.

This threefold sequence is strikingly similar to deposits surrounding the Chicxulub crater⁴, which was caused by the impact held responsible for the mass extinctions at the Cretaceous–Tertiary (K/T) boundary. The K/T sediments also begin with a thick basal unit of coarse current-transported blocks; unlike the record of the Eltanin impact, however, these basal K/T deposits are usually mixed with ejecta from the impact crater. The second, overlying K/T unit is characterized by parallel- and cross-bedded sands, indicative of deposition both by currents and from suspension. The third unit is a fine, graded-suspension deposit, and, like that seen in the *Polarstern* cores, it contains fine debris from the impacting body (in the form of an iridium anomaly and magnesioferrite spinels⁵). In particular, the grain size distribution of the topmost K/T unit at Brazos River, Texas⁶, is similar to that in the top unit of the Eltanin impact deposits; both represent the final settling of fine, resuspended sediments.

What might have been the further consequences of the Eltanin impact? It would have caused huge tsunamis, which would have hit the South American coast and run hundreds of metres inland leaving sandlayers with marine elements far away from the coastline. Such deposits, however, are easily eroded and thus have probably vanished (although the 'bonebeds' near Pisco in Peru, which contain mixed marine and terrestrial vertebrate skeletons of late Pliocene age, may form the remains of one). Other evidence of huge tsunamis resulting from large oceanic impacts should be found in near-coastal areas, below the storm wave base. Such deposits probably occur frequently in the geological record, but are not recognized or are interpreted as unusual turbidites, coastal sands or lowstand deposits rather than tsunami deposits.

So there is much scope for further detective work. Statistics tell us that some 500 extraterrestrial bodies of similar size to the Eltanin meteorite hit the oceans during the Phanerozoic (that is, over the past 540 million years). Where might the traces of these events be hiding? □

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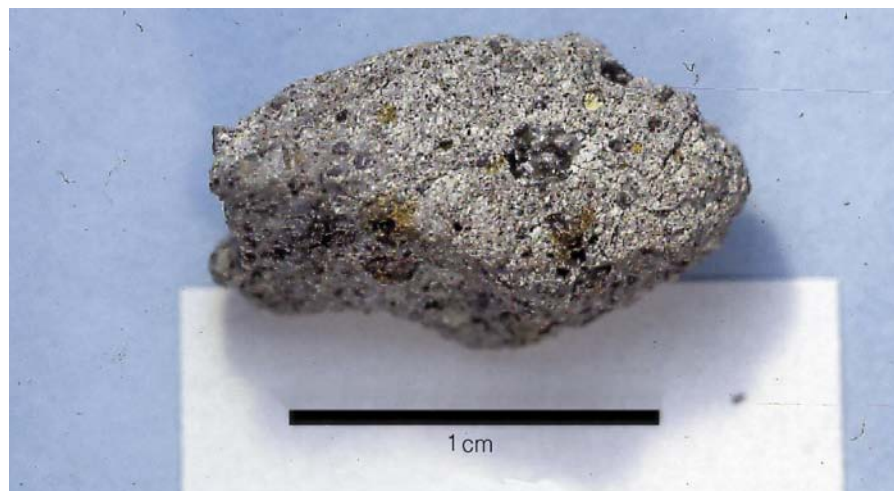


Figure 1 The largest unmelted remnant of the Eltanin meteorite recovered by FS *Polarstern*. It is a fragment of mesosiderite, a rare class of differentiated, stony–iron meteorite, and comes from core PS2708-1 (that drilled at intermediate depth; see Fig. 3 on page 359). (Photo courtesy of R. Gersonde.)

Evolutionary genetics

Unravelling gene interactions

Sarah P. Otto

Genome sequencing projects have generated a flood of information about the molecular basis of life. As the catalogue of genes has grown, so too has our understanding of gene function. For example, of the 4,288 protein-coding genes in the bacterium *Escherichia coli*, 62 per cent have been assigned to a functional class¹. Yet we remain quite ignorant about how these genes work together to create a whole organism. Understanding how genes interact is important in its own right but also has profound implications for evolutionary biology².

On page 395 of this issue³, Elena and Lenski provide the clearest picture to date of the ways in which genes, randomly chosen from throughout the genome, interact to affect fitness. They inserted a known number of transposable elements into random positions of the *E. coli* genome, and calculated growth rate over a six-day period for bacteria with different numbers of mutations. If the function of a gene relies on a complex web of interactions, the effect of a mutation on fitness will depend on which other mutations are present. In this case, evolutionists say that there is 'epistasis' between the genes, because the effects of mutations are not independent of one another. In these experiments, however, the authors found that, on average, each additional mutation slowed the rate of cell division by roughly the same amount, suggesting that epistasis was not present.

Just as one settles into the comfortable interpretation that random mutations affect such different aspects of cell performance that they are effectively independent, Elena and Lenski demonstrate that there are interactions between many pairs of mutations. In an additional set of experiments, they created recombinant *E. coli* with different pairs of mutations and compared them to parental strains carrying each of the mutants separately. In 14 out of 27 cases, the fitness of the double mutant was significantly different

from that expected from the product of the single-mutant fitnesses (the probability of observing so many significant differences is $<10^{-11}$). No epistasis was observed on average because the pairs of mutations ameliorated each others' effects about as often as they exacerbated them. This result is remarkable and has a number of implications.

With interactions between fully half of the pairs of mutations, we must re-evaluate the importance of gene interactions. Why would a pair of genes randomly chosen from a genome be sensitive to each other's action? This result cannot be explained by the existence of protective mechanisms that break down as more mutations accumulate (mutations would then only exacerbate one another's effects). Perhaps enzymatic pathways and regulatory networks are longer, more interconnected and more sensitive to structural changes in the cell than is often appreciated.

Indeed, this explanation is consistent with an experiment in *Drosophila melanogaster*, where characteristics such as protein, glyco-gen and fat content, and body weight and enzyme activities, were examined in lines containing transposable-element insertions⁴. Epistatic interactions were rampant, with insertions often affecting several characteristics. It is also consistent with the observation⁵ of great variability in the form of epistasis between pairs of genes, because different epistatic relationships are expected to occur within different enzymatic pathways⁵. The availability of the entire genome of *E. coli*¹ should make it possible to identify the genes affected in such experiments and, for many of them, their putative function. We will then be able to determine more precisely the nature of epistatic interactions.

The observation that genetic interactions are common but highly variable will have an even greater impact on the evolutionary theory of sex and recombination. One of the more popular theories for why sexual repro-

duction is ubiquitous, despite being a risky and costly mode of reproduction, is that sex allows deleterious mutations to be eliminated more efficiently from a population (the mutational deterministic hypothesis^{6,7}). Sex only facilitates the elimination of deleterious mutations, however, when fitness decreases faster than predicted with each additional deleterious mutation, a phenomenon called synergistic epistasis (see Fig. 1; refs 8–10). If each additional mutation has less and less of an impact on fitness (antagonistic epistasis), then sex actually hinders the elimination of deleterious mutations. The fact that epistasis was not predominantly synergistic in the new experiments³ therefore contradicts the main requirement of the mutational deterministic theory for sex. Even if synergistic epistasis were present, sex and recombination need not evolve. Variance in the extent of gene interactions, as found by Elena and Lenski³, reduces selection for sex and recombination (see Fig. 1; ref. 9). So the absence of synergistic epistasis and the great variance in epistatic interactions observed are double blows to the mutation-elimination hypothesis for sex and recombination.

One might object that, after all, *E. coli* bacteria rarely exchange genetic material¹¹ and may not be subject to the type of genetic interactions typical of sexual organisms. There is, however, no obvious reason why genetic interactions in sexual eukaryotes should necessarily differ from those in *E. coli* — all cells must perform housekeeping tasks and use similar, often homologous, genes to do so. Perhaps sexual eukaryotes have evolved elaborate protective mechanisms that minimize the effects of a few mutations but fail under the burden of many mutations (resulting in synergistic epistasis). Or perhaps sexual eukaryotes face different metabolic demands compared with bacteria, such that the balance between enzyme pathways is altered and synergistic epistasis is made more common⁷. Studies similar to Elena and Lenski's, but in a number of different species, are needed to determine the nature and variety of genetic interactions — information that is of fundamental importance to both molecular and evolutionary biology. □

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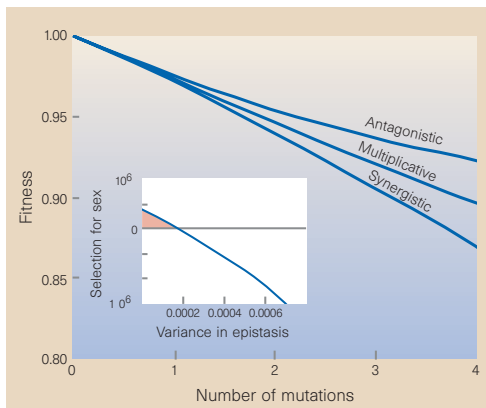


Figure 1 Fitness versus the number of deleterious mutations. The main figure shows fitness as a function of the number of deleterious mutations under conditions of synergistic, multiplicative and antagonistic epistasis. The inset shows the strength of selection favouring an increased amount of sex as a function of the variance in epistasis (calculated from ref. 9), assuming that, on average, there is synergistic epistasis (necessary for sex to be advantageous). Sex and recombination are able to evolve only when there is little variability in the amount of epistasis (shaded area).

Materials science

Silicon self-analysis

Robert W. Cahn

In antiquity, the Greeks invented scientific theory. Then, in the late sixteenth century, there were the first stirrings of proper scientific experiment. Finally, in the last third of the twentieth century, computer simulation arrived, and by now the three categories have achieved equal prominence. One active coven of simulators, at the Argonne National Laboratory in the American Midwest, has just succeeded in casting new light on an old problem, the structure of grain boundaries in a polycrystalline assembly. Keblinski *et al.*¹ have published persuasive evidence that a certain kind of high-energy grain boundary in simulated polycrystalline silicon consists of a glassy (amorphous) layer in thermodynamic equilibrium.

This is a truly remarkable conclusion to reach. But it comes almost a century after the originator of the science of physical metallurgy, Walter Rosenhain, first proposed the idea that grain boundaries in metals are amorphous^{2–4} (and strengthened thereby) — an idea that was met by large-scale scepticism at the time and has been dismissed altogether more recently. So has Rosenhain been vindicated?

Rosenhain cited two slightly earlier studies. One proposed that work-hardening in metals is due to the creation of an amorphous layer within slip bands; the other showed experimentally that a mechanically polished surface layer on a metal has liquid-like characteristics. Neither of these features could be stable; either would be destroyed by a high-temperature anneal, as Rosenhain recognized. But he believed his postulated intergranular amorphous layer to be stable up to the melting temperature.

Any glassy metal, we know today, will crystallize when heated. But the idea of a thermodynamically stable glass is acceptable for covalent silicon, a material in which the amorphous state withstands heating up to a well-defined melting temperature, as does crystalline silicon⁵. So, Rosenhain has been vindicated ... but only up to a point.

Rosenhain's model for grain boundaries in metals⁶ has been dismissed mainly because experiment has shown that the specific energy of a grain boundary increases as the misorientation of the two grains goes from nothing to about 15–20°; and it is clear that if all grain boundaries consisted of similar glassy layers (which can have no inherent directionality), this fact could not be explained. So there must be a transition region with a fine structure that varies with the degree of misorientation^{6–8}.

No such energy measurements appear to have been made for grain boundaries in

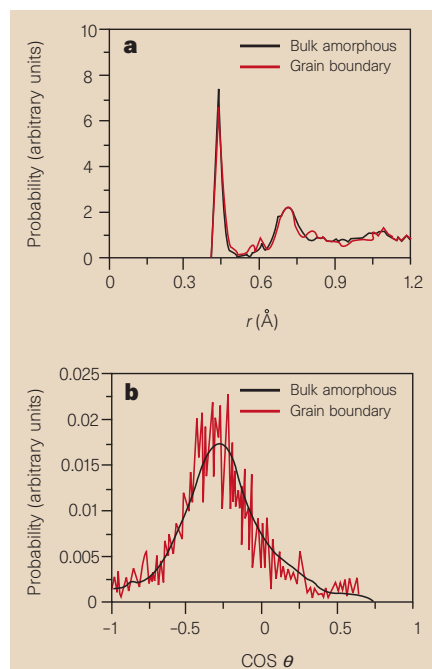


Figure 1 Evidence of amorphous material at silicon grain boundaries.

The distribution of atomic separations, in the form of a radial distribution function, of the most highly disordered plane in the region of a high-misorientation grain-boundary on the

silicon, but it is a reasonable assumption that, as in metals⁸, for large misorientations the boundary energy no longer depends appreciably on misorientation. In an earlier paper⁹, Keblinski and colleagues simulated large-angle boundaries where the orientations differ by rotation about an axis normal to the boundary ('twist' boundaries), which they believed to be more energetic than large-angle 'tilt' boundaries, where the rotation axis lies in the boundary plane. Their results of this simulation of individual grain boundaries were much the same as those in their new paper on grain boundaries in polycrystalline microstructures¹, in which they allow several grains to crystallize from random nuclei in molten silicon and grow until they abut one another.

For their simulations, Keblinski *et al.* used a 'three-body potential' for silicon¹⁰; this potential apparently provides a good description of the diamond-structure crystalline form, and also offers an accurate estimate of the structure and excess energy of the amorphous bulk form. This approach was used in preference to a less empirical tight-

binding approach¹¹, because it permits simulations on large numbers of atoms (as many as 100,000). The main conclusion is that for large-angle (and thus high-energy) boundaries in silicon, the introduction of an amorphous layer ~ 0.25 nm thick into a large-angle crystalline boundary lowers the energy. Equivalently, the stable boundary structure derived from the simulations has characteristics that mark it as amorphous (in Fig. 1 the boundary characteristics are compared with those of amorphous silicon). So an amorphous layer is thermodynamically stable in a large-angle crystalline boundary — whereas for a small-angle boundary, a crystalline interface is stable.

These findings imply¹² that silicon, unlike a range of ceramics in which very thin amorphous layers have been observed experimentally¹³, needs no dissolved impurities to explain the stability of the amorphous layer. The conclusion that high-energy grain boundaries really are amorphous in thermodynamic equilibrium has been vindicated by the team's latest simulations¹⁴, in which they demonstrate "the existence of a reversible structural and dynamical transition from a confined amorphous solid to a confined liquid". The fact that amorphous grain boundaries in silicon have not yet been reported by electron microscopists is perhaps to be explained by the lack of contrast between amorphous and crystalline regions in silicon.

This sequence of simulation 'experiments' is a convincing demonstration of the power of the approach in settling difficult scientific questions. As stated at the outset, computer simulation is *sui generis* — neither pure theory nor pure experiment. It is intriguing to speculate what Walter Rosenhain, that superb experimentalist and combative would-be theorist, would have made of the Argonne work. □

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Muscle contraction

Myosin trapped but not tamed

Clive R. Bagshaw

Muscle shortening involves interdigitation of actin- and myosin-containing filaments driven by the action of cross-bridges powered by ATP hydrolysis. The detailed mechanism remains in question, however, and current research has centred on definition of the structures of the cross-bridge (the head of the myosin molecule) and actin at atomic resolution, and the development of assays to follow mechanics and chemistry at the single-molecule level. At the annual European conference on muscle*, new developments were announced in these areas, along with progress on other topics, including fresh ways of looking at muscle disease.

The myosin molecule from muscle is composed of two pear-shaped heads, which interact with actin and contain the ATP-hydrolysing sites, attached to a long coiled-coil tail by 8-nm neck pieces, known as the regulatory domains. The dogma is that, at some point during the ATP hydrolysis cycle, a myosin head bound to the actin filament changes its attachment angle or bends at the head-neck junction to sweep out a displacement of about 10 nm at the distal end of the regulatory domain. X-ray crystallographic studies have attempted to define the structures of intermediates of the ATPase cycle by using analogues which trap the nucleotide at different points in the cycle. Structures have been defined¹ in the presence of ADP with beryllium (BeF₃) or aluminium fluoride (AlF₄), which substitute for the γ -phosphate and hold the nucleotide in a long-lived bound state (chemical trapping). These are proposed to mimic solution states of myosin corresponding to M.*ATP and a transition approaching M.**ADP.P_i with BeF₃ and AlF₄, respectively.

New crystal structures presented at the meeting show that non-hydrolysable or slowing hydrolysable ATP analogues (such as AMPPNP and ATP γ S) fall into the M.*ATP class, but perhaps more surprisingly, so does the myosin catalytic domain with a vacant active site (A. Gulick, Univ. Wisconsin). Indeed, this apo crystal form can take up ATP with little change in structure. So there is good news and bad. We have a crystal structure for bound ATP, but one which cannot undergo hydrolysis, presumably because crystal forces prevent the head from making the required conformational shifts.

To add to this conundrum, the trapped M.ADP.BeF₃ state crystallized in Heidelberg (K. Holmes, MPI for Medical Research) is more comparable to the structure of the

M.ADP.AlF₄ structure from Wisconsin. Although relating nucleotide states and structural states remains a challenge because of the influence of crystal forces, it is consoling that the differences between the two structural classes involve shifts in the residues at the bottom of the phosphate-binding pocket which are closely analogous to the switch observed in G proteins as they convert from the GTP to GDP states. Thus these movements appear to be physiologically relevant.

The myosin fragments whose crystal structures have been solved to date, in the presence of nucleotide, unfortunately lack the regulatory domain. But there are structural differences at their carboxy termini which suggest that the neck would emerge at a different angle in the two states defined above. Consequently, the regulatory domain attached to this region could act as a lever to amplify small (0.5-nm) conformational shifts observed in the crystal structures into 'macroscopic' steps of the order of 10 nm for each round of ATP hydrolysis.

In vitro motility assays, where single actin filaments are observed to slide over immobilized myosin, support this idea. Using engineered constructs, it was found that the longer the lever, the faster the myosin drives actin filament sliding², but there is nothing like seeing the lever arm bend directly. Tantalizing electron micrographs of skeletal-muscle myosin heads containing the regulatory domain indicate ADP binding favoured a bent conformation over the gently curved structure of the apo form (A. J. Rowe, Univ.

Leicester). This contrasts with studies of the head bound to actin where such a transition with ADP was only seen for smooth muscle myosin³.

The 'bending head' model most neatly fits in with a 1:1 coupling between the mechanical and chemical cycles in which one sweep of the neck consumes one ATP molecule. Experiments involving simultaneous use of optical trapping and total internal reflectance fluorescence microscopy provide direct evidence for a close correspondence between mechanical events and nucleotide turnover at the level of a single myosin head (T. Yanagida, ERATO project, Univ. Osaka; Fig. 1). Although the binding of a fluorescent ATP analogue triggered the simultaneous release of the actomyosin bond (within the 0.1-s time resolution), the rebinding of actin to initiate displacement of the trap often occurred 0.2 to 0.8 s after nucleotide had apparently dissociated.

In consequence, Yanagida speculates that myosin has a hysteretic state which stores energy from ATP hydrolysis. The concept that actomyosin bond formation can perform work is not so radical, because bond formation is two-step⁴ and could involve a stereospecific rearrangement following binding. However, Yanagida countered this simple explanation with his finding that displacement transients generated in the absence of nucleotide averaged to zero, and thus yielded no net movement.

The interest in this coupling mechanism has been at the forefront of research following the possibility of long (>100 nm) interaction distances per ATP hydrolysed, deduced from *in vitro* sliding assays which would require multiple strokes of the cross-bridge or some kind of energy storage in actin⁵. The close correspondence

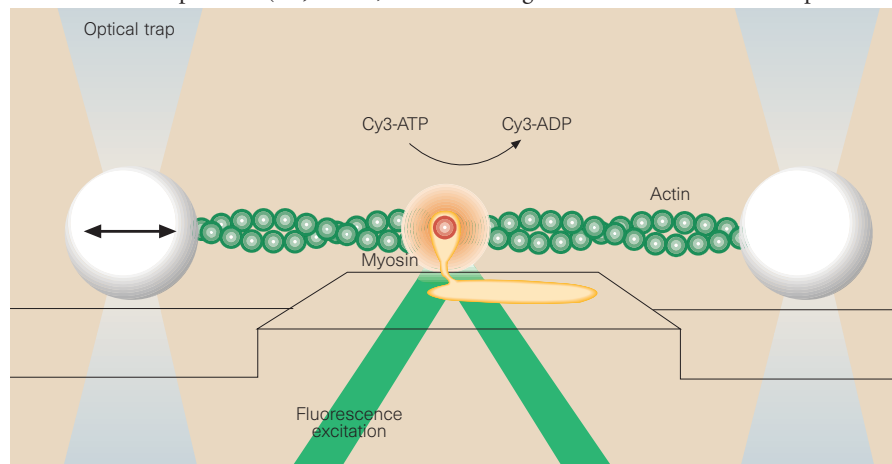


Figure 1 Simultaneous measurement of ATPase and mechanical displacement by a single myosin head. An actin filament is held by two beads in an optical trap and brought to within the interaction distance of a single myosin head in a sparse myosin-rod copolymer. Mechanical displacements on the nanometre scale are monitored by deflection of the beads, while the nucleotide binding and release during ATP turnover are monitored using a fluorescent analogue, Cy3-ATP, and evanescent excitation by total internal reflection fluorescence microscopy. (Modified from a graphic provided by T. Yanagida.)

*XXVI European Muscle Congress, Schneverdingen, Germany, 21–25 September 1997. Abstracts to be published in *J. Muscle Res. Cell Motility*.

between chemical and mechanical steps, and an upper limit of unitary displacements of 30 nm now reported (Yanagida), leaves the controversies of the sliding assay unexplained; if correct, it implies that arrays of myosin heads are required for long interaction distances.

Myosin's cousins in the kinesin family also came in for discussion. Kinesins are motor proteins which interact with tubulin tracks to power a variety of intracellular transport processes. Some of its members operate in a highly processive manner, giving experimenters more chance to characterize attached states than in the actomyosin system (where nucleotide binding tends to dissociate the protein complex). However, the similarities in the overall structures of the kinesin and myosin heads suggests some features of the mechanochemical coupling mechanism might be the same.

The crystal structure of a rat-brain kinesin head domain (E. Mandelkow, DESY, Hamburg) reveals details of the amino and carboxy termini not visible in previous studies⁶. These regions are important because the orientation of the α -helical neck is now defined and it seems inappropriate for the neck to act as a lever analogous to the myosin model. Furthermore, if both heads of kinesin are able to attach to tubulin subunits 8 nm apart, the neck region would need to unravel (R. Cross, Marie Curie Inst., Oxted, Surrey). The functions of the neck regions in myosins and kinesins therefore appear to be very different.

Finally, at the meeting clinicians and scientists came together to look at ways of exploiting natural mutations to understand protein function and, maybe, to develop more precise diagnostic tools for identifying specific molecular defects underlying muscle disease. For example, mutations in ion channels and pumps lead to aberrant activation which can trigger malignant hyperthermia (A. Lehmann-Horn, Univ. Ulm; D. H. MacLennan, Univ. Toronto), and mutations in certain contractile proteins cause familial hypertrophic cardiomyopathy (W. J. McKenna, Univ. London; H. Watkins, Univ. Oxford). The latter condition can result in sudden death during strenuous exercise, but it is actually more than one disease in that the underlying defect may involve mutations in the myosin heavy chain or light chains, tropomyosin or C-protein⁷.

Generally, familial hypertrophic cardiomyopathy is associated with enlargement of the heart (a compensatory mechanism which attempts to maintain pumping capacity). But the age of onset and degree of hypertrophy varies in individuals carrying the same mutation and, in itself, is not a reliable diagnostic tool. Identification of the condition therefore requires genetic screening. Of particular interest, however, is that

some people are able to carry a potentially lethal mutation with little effect on their physiology and have a normal lifespan, which suggests that compensatory mechanisms are at work. □

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Cosmology

The case of the missing ellipticals

Guinevere Kauffmann

Galaxy formation: early or late? This is one of the most hotly debated questions in cosmology. Some believe that galaxies have evolved through a complex series of interactions over most of the age of the Universe and have only settled into their present-day form recently. Others believe many galaxies were created in a well-defined event at a very early time. Both sides agree, however, that it is the elliptical galaxies

— the oldest and most luminous stellar systems in the Universe — that may soon resolve the question of how and when the first galaxies formed.

On page 377 of this issue¹, Zepf reports the discovery that fully formed elliptical galaxies are rare at high redshifts (that is, at great distances, and therefore at early times). These findings were made possible in part by a very deep snapshot of the sky, the Hubble Deep

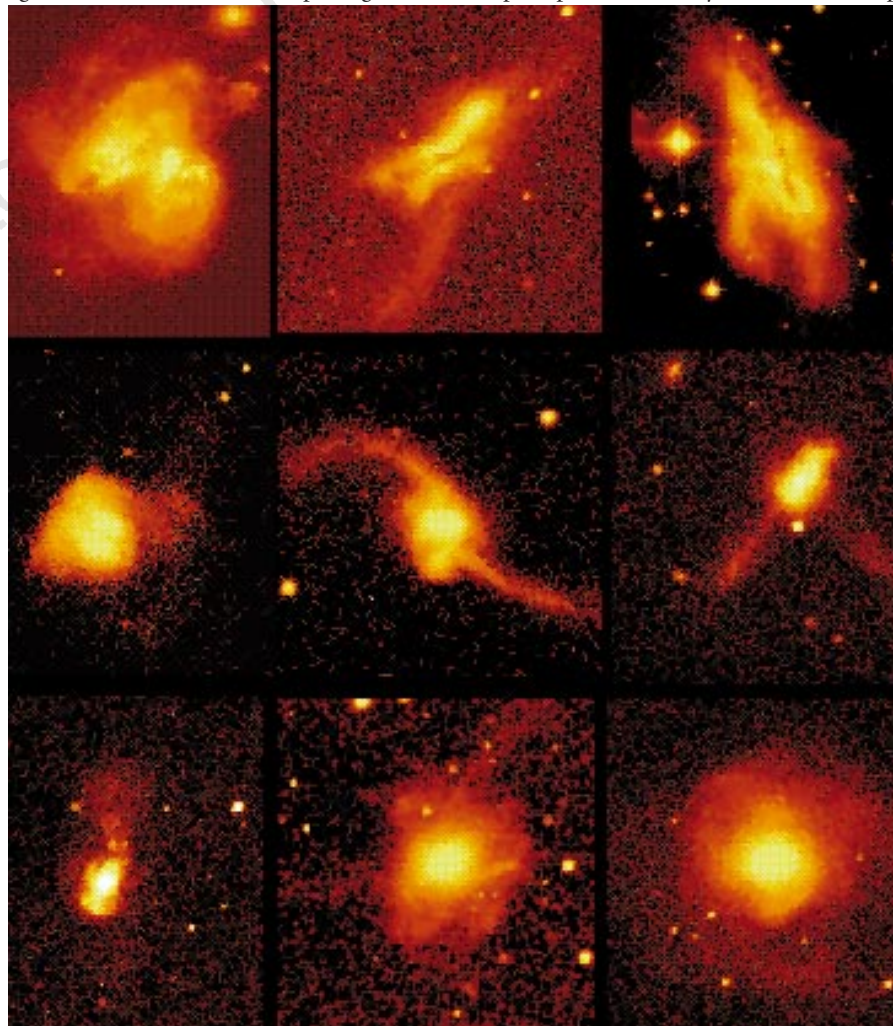


Figure 1 'Merger movie' — nine nearby pairs of merging galaxies are arranged in approximate time order (the first pairs are only just beginning their mergers, and the last pairs have just about finished). The rarity of elliptical galaxies in the early Universe supports the idea that most ellipticals are formed in mergers.

BILL KEEL

Field, taken in December 1995 by the Hubble Space Telescope. For two weeks, the telescope focused on a single region of sky, allowing unprecedentedly faint galaxies to be imaged. This provided astrophysicists with an extremely powerful time machine—the most distant galaxies in the Hubble Deep Field are so far away that light from them has spent more than 10 thousand million years travelling to reach us². We observe these galaxies as they were when the Universe was only a tenth of its present age. The relationship between these very early objects and present-day galaxies remains a mystery, however.

Zepf realized that if elliptical galaxies exist at high redshift in much the same form as in the present-day Universe, they ought to be easily picked out of deep surveys like the Hubble Deep Field. The observational quantity required to do that is the ratio of optical and infrared brightness: the ‘optical–infrared colour’ of a galaxy is said to be red if the ratio is small; blue if it is large. Galaxies that have stopped forming stars are red, because hot, blue, massive stars quickly explode and die, leaving behind a population of cooler, less massive stars that emit most of their light at infrared, rather than optical wavelengths.

Using the Hubble Deep Field (at optical wavelengths) and data sets from infrared cameras on telescopes on the ground, Zepf finds that galaxies with colours characteristic of present-day ellipticals are almost completely absent from surveys of faint galaxies. In particular, there are far fewer very red galaxies than one would expect if the present-day population of elliptical galaxies had formed by a redshift of five.

Why are the missing ellipticals so exciting for cosmologists? The answer has to do with arguments about how these objects formed. Many cosmologists now believe that at high redshifts only low-mass galaxies existed—the building blocks of the galaxies we see today. As time went on, many of these small proto-galaxies coalesced under the influence of gravity, gradually forming the more massive galaxies that populate our corner of the Universe. This model is so popular among theorists that it is often referred to as ‘the standard model of structure formation’. There is no question that galaxies do occasionally collide with one another and merge (Fig. 1).

Many famous cases are known, including an interacting pair called the Antennae, where tidal forces have ripped huge plumes of gas and stars out of the galaxies and spewed them into space. There is also strong, though more indirect, evidence from computer simulations that the remnants produced by such mergers will eventually settle down to look much like elliptical galaxies³.

Yet many other astronomers doubt

whether the majority of elliptical galaxies could have been formed by mergers. They cite the very great stellar ages, the high abundances of certain chemical elements and the uniformity of these objects (in colour, and in luminosity for a given size) as evidence that ellipticals formed very early and over a short period of time. This is called the single-burst model.

The reader should be cautioned that the fact that red galaxies appear to be missing in deep surveys does not yet spell victory for the merger model and defeat for the single-burst model. Zepf discusses two ways in which ellipticals formed in a single burst could be hidden from view, or simply avoid detection. One possibility is that ellipticals are being produced in dusty starbursts at redshifts less than five. Large amounts of dust can absorb radiation emitted by the forming galaxy, obscuring it from view. Another possibility is that ellipticals already exist at high redshifts, but continue to form stars at a low, but not vanishing, rate. The extra light produced by only a few massive stars would make a galaxy too blue to meet Zepf’s criterion for selection as an elliptical.

Observations will undoubtedly settle these questions soon. Dust absorbs high-frequency radiation, but re-emits the energy at far-infrared and sub-millimetre wavelengths. Far-infrared surveys of the sky designed to detect dusty star-forming galaxies at high redshift are already under way^{4,5}. It should also become obvious if high-redshift ellipticals are indeed out there, but unrecognized because of gentle star formation, once redshifts have been measured for large samples of distant galaxies.

It must surely be a sign of the vitality of a subject if so many answers are in the offing after so many years of debate. Readers, stay tuned for the next episode in the case of the missing ellipticals. □

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Erratum

In the News and Views article “Galaxies take the distance record” by Kenneth Lanzetta (*Nature* **390**, 115–116; 1997), the author’s address was omitted. Kenneth Lanzetta is in the Department of Earth and Space Sciences, SUNY at Stony Brook, Stony Brook, New York 11794-2100, USA.

Daedalus

Shrinking or swelling?

Last week Daedalus pointed out that our body fat, like that of all organisms, is stored in liquid form. He proposed an ‘oligomolecular diet’ of a few well-chosen fats, whose mixture melted only just below our core body temperature. In the body it would act as thermal ballast, giving out latent heat of solidification whenever its owner was exposed to cold.

He now wonders why we store our fat as a melt in the first place. The obvious answer is that biochemistry is a liquid-phase business. A solid fat cannot be digested, mobilized or metabolized. So he now sees his high-melting oligomolecular fat as a powerful new weapon in the war against obesity.

Many dieters complain that their efforts to lose weight remove fat from exactly the wrong parts of the body. Women, for example, may shed fat from their faces (making them look lined and anxious) or from their busts; men may shrink their thighs to emaciation while retaining a corpulent paunch. But the user of DREADCO’s oligomolecular fat could simply put a cold pack on those regions that he or she wished to retain. The fat beneath would solidify, and biochemistry could not touch it. Other, less becoming regions would be depleted instead. At the cost of some thermal discomfort, the dieter could lose weight in all the right places, and none of the wrong ones. Better still, solidified fat should be very firm and resilient. Many people dislike their bodies, not for their size, but for their flabbiness. Oligomolecular fat should give a lean, taut appeal even to a fairly ample figure.

This hopeful conclusion is dogged by a converse argument. If solidified fat ceases to register on the body’s inventory, the body’s internal weight-stat may decide that there is no fat in that deposit at all. It will hasten to lay down new fat, which of course will solidify and vanish from view in its turn. The user will gain weight endlessly in the cooled region. Daedalus recalls that immigrants to northern parts from hot areas often become grossly obese. Perhaps their fat, optimized for the tropics, simply solidifies? If so, the traditional steam-room or Turkish bath may indeed be an effective slimming treatment; and the dietary benefits of low-melting polyunsaturated fats will be confirmed. Either way, varying the melting-point of our body fat seems to offer ways into the problem of obesity. Overweight DREADCO volunteers are now putting the matter to the test.

David Jones

Stubbs' seeing

The English painter George Stubbs, best known for his pictures of horses, was recognized in his own lifetime for the anatomical exactitude of his work. His reputation led him into the debates about creation and extinction.

Martin Kemp

The revolution in visual communication in the Renaissance nowhere exercised a more profound effect than on the illustration of natural history. Although skill in naturalistic representation can be a double-edged sword — an accomplished artist can make a unicorn look as 'real' as a narwhal — the rigorous portrayal of specimens from life played a key role in conveying knowledge about the ever-widening spectrum of natural marvels. That most English of English painters, George Stubbs, stood as a distinguished heir to the Renaissance tradition and demonstrates vividly how pictorial evidence can serve as powerful tool for both information and speculation.

In 1770, probably in the autumn, the physician in ordinary to Queen Charlotte, William Hunter, "obtained leave to have a Picture made... by Mr. Stubbs" of the first bull moose to arrive from Canada, imported by the Duke of Richmond. As Hunter tells us, "no pains were spared by that great Artist to exhibit an exact resemblance both of the young animal itself and of a pair of Horns of the full-grown Animal".

Hunter's desire to pin down the exact appearance of the moose was motivated less by old-fashioned curiosity than by the newest debates about extinction.

Three years later he inspected a second moose imported by the Duke, "carrying with us Mr. Stubbs's picture".

Hunter had already argued conclusively that the bones of the so-called Ohio incognitum

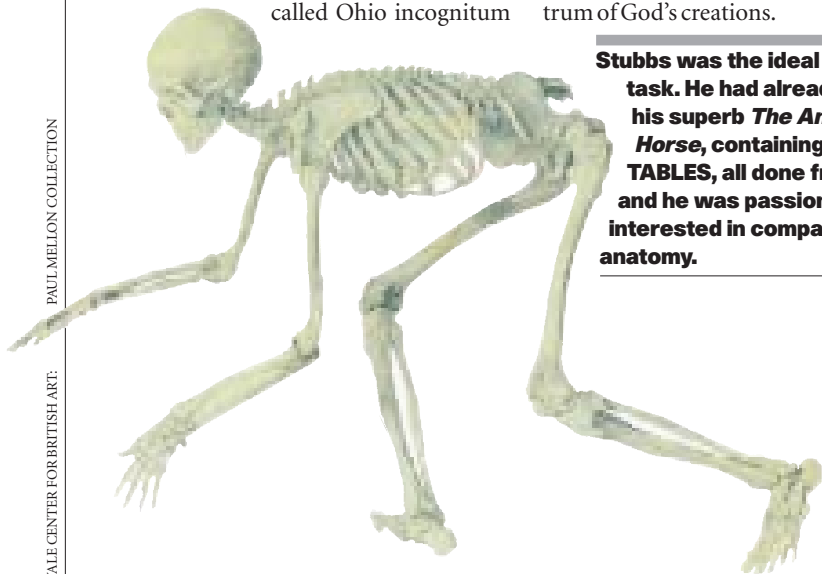


George Stubbs, *The Duke of Richmond's Bull Moose*, 1770.

(the mastodon) were not those of an elephant, and he intended Stubbs's painting to confirm that the 'Irish Elk', known through fossils, was not the same as the extant moose. Such a confirmation would have broken another of the links in 'the Great Chain of Being', the traditional doctrine that maintained the eternal continuity of the full spectrum of God's creations.

Stubbs was the ideal artist for the task. He had already published his superb *The Anatomy of the Horse*, containing "eighteen TABLES, all done from Nature", and he was passionately interested in comparative anatomy.

George Stubbs,
The Human Skeleton in Crawling Posture, c.1800.



The project on which he was working at his death in 1806 was *A Comparative Anatomical Exposition of the Structure of the Human Body with that of a Tiger and a Common Fowl*, for which he drew some remarkable images of a human skeleton in animal postures.

His paintings of wild animals, particularly those of terrified horses savaged by lions, purveyed an innovative view of the dramatic interplay of animate and inanimate agencies in nature.

Even if his static moose does not lend itself to exciting narrative, the stirring nature of the lakeland wilderness inhabited by the creature is enhanced by the febrile gloom of the encroaching storm.

Hunter never published his paper on the 'Irish Elk', in the absence of secure data about the range of antler types in the mature moose and uncertainty about the possible survival of the 'Elk' in as yet unexplored wastes. But the artist and the doctor were beginning to sow the intellectual seeds that Darwin was to cultivate to such effect in the nineteenth century.

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Melanocortin receptors in leptin effects

Leptin acts on the central nervous system to cause a reduction in food intake and body weight^{1,2}. The melanocortin system in the brain is also implicated in energy homeostasis, with agonists of the melanocortin-4 (MC4) receptor reducing food intake³ and targeted mutation of the MC4 receptor causing obesity⁴. We now show that MC4 receptor signalling is an important mediator of leptin's effects on food intake and body weight, demonstrating a link between the two systems.

Leptin receptors are on proopiomelanocortin (POMC) neurons in the arcuate nucleus⁵ that synthesize melanocortins, therefore it seemed likely that leptin-induced reductions in food intake might be mediated by the melanocortin system. To test this hypothesis, we determined whether pharmacological blockade of MC4 receptors attenuates the ability of leptin to reduce food intake or body weight. Because leptin also activates the hypothalamic paraventricular nucleus (PVN), as measured by c-Fos expression, we also wished to determine whether this response involves melanocortin receptors.

We injected male Long-Evans rats with the non-selective MC4 receptor antagonist SHU9119 into the third cerebral ventricle (i3vt). There was a significant increase in food intake 4 and 24 h after administration of 1 nmol SHU9119, whereas 0.5 nmol altered neither food intake nor body weight at any time point. We therefore pretreated rats with 0.5 nmol of SHU9119 or vehicle i3vt before an i3vt injection of 3.5 µg of recombinant leptin or its vehicle (see ref. 1 for details). Consistent with previous findings¹, leptin alone significantly reduced food intake after 4 h (−54%) and after 24 h (−46%, $P < 0.05$; Fig. 1a), and reduced body weight after 24 h (−18.6 g, $P < 0.05$). However, when these same animals were pretreated with SHU9119, leptin did not affect food intake or body weight.

To assess the specificity of SHU9119 to reverse anorexia induced by leptin, we administered glucagon-like-peptide-1 (7–36) amide (GLP-1), a potent inducer of anorexia when given intracerebroventricularly^{6–9}. The dose of GLP-1 (10 µg i3vt) that we selected

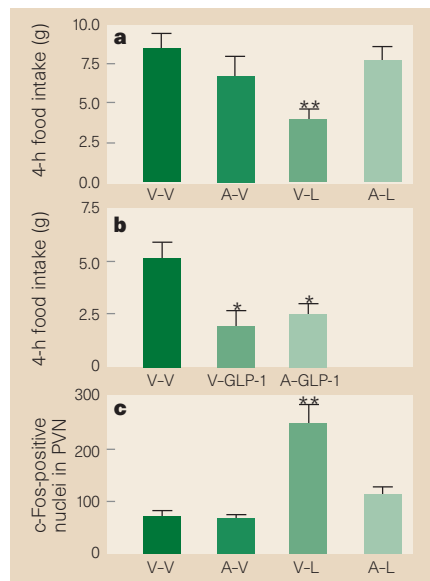


Figure 1 Effect of treatment with a melanocortin receptor antagonist on feeding and the leptin response (means±s.e.m.). V, vehicle; A, antagonist; L, leptin. Data were analysed by a one-way ANOVA followed by post-hoc Neuman-Keul's tests. * Significant difference from the vehicle-only group at $P < 0.05$; ** at $P < 0.01$. **a**, Effect of pretreatment with i3vt SHU9119 (0.5 nmol) on the anorexic response to i3vt leptin (3.5 µg) on 4-h food intake. Leptin treatments, $n = 12$; vehicle treatments, $n = 6$. **b**, The effect of pretreatment with i3vt SHU9119 (0.5 nmol) on the anorexic response to i3vt GLP-1 (10 µg) on 4-h food intake. GLP-1, $n = 5$; vehicle group, $n = 4$. **c**, Effect of pretreatment with i3vt SHU9119 (0.5 nmol) on c-Fos activation in the PVN of the hypothalamus produced by i3vt leptin (3.5 µg) treatment. Vehicle-only, $n = 5$; antagonist-vehicle, $n = 4$; leptin-treated groups, $n = 6$.

reduces short-term food intake to an extent comparable to 3.5 µg leptin⁷ (−63% after 4 h) and this effect was not altered by pretreatment with 0.5 nmol SHU9119 (Fig. 1b).

We assessed c-Fos-like-immunoreactivity in the PVN after leptin treatment with or without SHU9119 pretreatment (see ref. 10 for methods). Consistent with our previous findings¹⁰, leptin increased c-Fos-like immunoreactivity in the PVN by 254%, and this was completely blocked by SHU9119 (Fig. 1c).

Our findings provide direct evidence that MC4 receptor signalling is important in mediating leptin's effect on food intake and body weight. We suggest that obesity stemming from disrupted MC4 receptor signalling (for example, MC4-R knockout and *agouti* mice) results from the loss of an important downstream target in the leptin signalling cascade. The possibility that the melanocortin system is one mediator of leptin's action in the central nervous system is particularly intriguing in light of the recent finding that human obesity and hyperleptinaemia are strongly linked to a region of chromosome 2 near the POMC gene locus¹¹. The hypothesis that polymorphisms of the POMC gene are associated with leptin resistance and obesity is consistent with our findings implicating melanocortins in the leptin signalling pathway, and may have important implications for the pathogenesis and treatment of human obesity.

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Transcriptional squelching re-examined

The introduction of a potent transcriptional activator into eukaryotic cells can paradoxically suppress the transcription of a co-introduced target gene^{1–3}. This so-called 'squelching' is thought to result from titra-

tion of one or more general transcription factors (GTFs)⁴, indicating that GTFs might be in limited supply within the cell^{5,6}. We now find that in mammalian cells, squelching is limited to episomal target genes, whereas genes integrated into cellular chromatin are immune.

During the process of designing more potent transcriptional activation domains for use in gene therapy, we constructed chi-

maeric transcription factors composed of the yeast GAL4 DNA-binding domain and activation domains derived from the herpes simplex virus protein VP16⁷ and the NF-κB p65 protein⁸. We tested the proteins on a target gene composed of a secreted alkaline phosphatase (SEAP) reporter under the control of a minimal human interleukin-2 (IL-2) gene promoter flanked by five GAL4 binding sites.

When we transiently introduced the

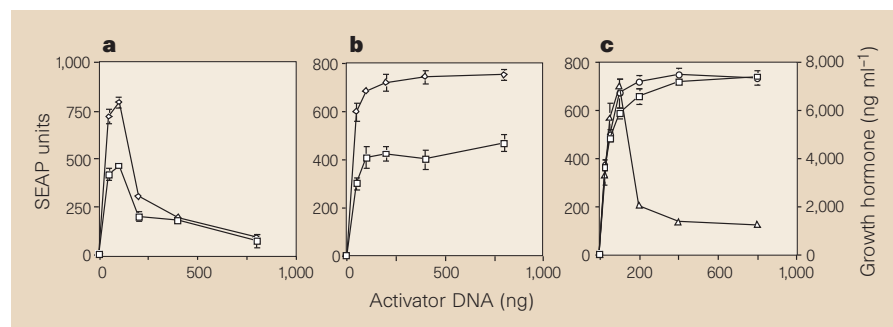


Figure 1 Transcriptional activation of genes embedded in chromatin and episomes. Increasing amounts of plasmids encoding chimaeric activator proteins GAL4-p65 (diamonds) and GAL4-VP16 (squares) were transiently transfected into either **a**, HT1080 cells cotransfected with reporter plasmid 5xGAL4-IL2-SEAP or **b**, HT1080B cells carrying the stably integrated reporter gene pLH-5xGAL4-IL2-SEAP. **c**, Increasing amounts of GAL4-p65 expression vector were transiently transfected into HT1080B cells in the presence or absence of co-transfected hGH reporter plasmid (5xGAL4-IL2-hGH). We measured the transcriptional activity of the integrated SEAP gene in the presence (squares) or absence (circles) of cotransfected hGH reporter plasmid, as well as the transcriptional activity of the transiently transfected hGH reporter plasmid (triangles). Mean values of SEAP activity and hGH protein secreted into the medium are shown ($\pm$ s.d.).

reporter gene and expression plasmids encoding the transcription factors into HT1080 human fibrosarcoma cells, we observed peak expression of reporter genes at relatively low levels of input activator plasmid, whereas higher levels of activator led to a sharp reduction in reporter gene expression (Fig. 1a). Such squelching in the presence of high levels of activator has been widely observed^{9,10}.

We tested the same transcription factors in an HT1080-derived cell line in which the same SEAP reporter gene had been stably introduced by retroviral transduction. Expression of the integrated reporter gene was not inhibited by levels of activator that produced pronounced squelching of a transiently transfected reporter (Fig. 1b). High intracellular concentrations of potent transcriptional activators cannot therefore be titrating GTFs required for transcription of an integrated chromatin-embedded gene.

It is possible that only DNA-bound activators effectively titrate GTFs. If so, the presence of high-copy episomal activator binding sites would be required for squelching to occur. We constructed a second reporter gene in which the same GAL4-driven IL-2 promoter was fused to a human growth hormone (hGH) reporter gene. This plasmid was cotransfected with the GAL4-p65 expression plasmid into cells containing an integrated SEAP reporter gene, thus allowing both reporter genes to be assayed in the same cell population. If, in the presence of a high-copy episomal template, GAL4-p65 titrates the GTFs necessary for activity of this promoter regardless of location, we would expect to see inhibition of both the episomal hGH gene and the integrated SEAP gene. Instead, although expression of the episomal hGH gene was inhibited at high activator concentrations, the integrated SEAP gene responded identically whether the episomal

gene was present or not (Fig. 1c). Thus, if the transcriptional activators are indeed titrating one or more GTFs required for transcription of the episomal gene, these GTFs are not required (or are not rate-limiting) for transcription of the integrated chromatin-embedded gene.

There are several possible explanations for our observations. Episomal and integrated genes may have different GTF requirements or rate-limiting steps for transcription. Limiting GTFs may bind with higher affinity to a chromatinized promoter, allowing chromatinized promoters to compete more effectively for GTFs. Episomal and integrated genes may reside in different compartments of the nucleus with independent GTF pools, or the conformational flexibility of an episomal DNA molecule may permit the formation of nonproductive transcription factor complexes that cannot form in the more constrained environment of chromatin. Whatever the explanation, the idea that GTFs are limiting for gene transcription in mammalian cells, an idea based largely on transient transfection assays, needs to be reconsidered.

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Immunosuppressive effects of apoptotic cells

Apoptotic cell death is important in the development and homeostasis of multicellular organisms¹ and is a highly controlled means of eliminating dangerous, damaged or unnecessary cells without causing an inflammatory response or tissue damage^{1,2}. We now show that the presence of apoptotic cells during monocyte activation increases their secretion of the anti-inflammatory and immunoregulatory cytokine interleukin 10 (IL-10) and decreases secretion of the pro-inflammatory cytokines tumour necrosis factor- α (TNF- α), IL-1 and IL-12. This may inhibit inflammation and contribute to impaired cell-mediated immunity in conditions associated with increased apoptosis, such as viral infections, pregnancy, cancer and exposure to radiation.

The lack of an inflammatory response during apoptosis has been attributed to the rapid phagocytosis of apoptotic cells before cell lysis, thereby preventing the release of noxious contents which could provoke inflammation and tissue damage². However, potent inducers of apoptotic cell death such as ultraviolet irradiation and X-rays ameliorate inflammatory diseases^{3,4}. We therefore investigated whether apoptotic cells simply fail to provide pro-inflammatory signals or whether they also actively suppress an inflammatory response.

Bacterial lipopolysaccharides (LPS) are potent stimulators of cytokine secretion from monocytes and macrophages. We tested whether apoptotic cells modify the LPS-induced cytokine secretion pattern of peripheral blood mononuclear cells (PBMC) and purified monocytes. In the presence of apoptotic peripheral blood lymphocytes (PBL), PBMC and monocytes produced substantially more of the anti-inflammatory cytokine IL-10 and less of the pro-inflammatory cytokines TNF- α , IL-1 β and IL-12 (Fig. 1). In contrast, living or freshly prepared paraformaldehyde-fixed PBL did not alter the cytokine secretion pattern (Fig. 1a,b and data not shown).

Various human and murine cells irradiated with ultraviolet light or γ -rays or deprived of growth factor had a similar anti-inflammatory effect on human monocytes, indicating that the anti-inflammatory properties of apoptotic cells are conserved between mammalian species and are independent of the apoptosis-inducing stimulus. The incubation time between monocytes and apoptotic cells before LPS stimulation influences the changes in the cytokine secretion pattern. Whereas the induction of IL-10 is highest without pre-incubation, the relative inhibition of pro-

inflammatory cytokine secretion increases with the preincubation time (Fig. 1c, d).

We tested whether surface receptors of monocytes and macrophages, which are involved in recognition and uptake of apoptotic cells, can also mediate anti-inflammatory signals. The thrombospondin receptor (CD36) which is expressed on various cells including monocytes and macrophages, dendritic cells and microvascular endothelial cells is involved in phagocytosis of apoptotic cells². The trimeric glycoprotein thrombospondin is believed to form a 'molecular bridge' between anionic sites on apoptotic cells and CD36 and the vitronectin receptor ($\alpha_5\beta_3$) on phagocytes^{2,5}.

Incubation of PBMC and monocytes with monoclonal antibodies against the thrombospondin-binding site of CD36 mimicked the anti-inflammatory effect of apoptotic cells (Fig. 1a, b). Further, thrombospondin-specific antibodies which block the engulfment of apoptotic cells decreased IL-10 and restored TNF- α , IL-1 β and IL-12 production by monocytes in the presence of apoptotic cells (not shown). Therefore, CD36-ligation by apoptotic cells may mediate their anti-inflammatory effect. Although the vitronectin receptor apparently does not transmit an anti-inflammatory signal (not shown), we cannot rule out the involvement

of other receptors besides CD36.

Cell-mediated immune responses are dependent on the production of interferon- γ (IFN- γ), which is induced by IL-12 but inhibited by IL-10^{6,7}. Increased IL-10 secretion and decreased IL-12 secretion by antigen-presenting monocytes and macrophages in the presence of apoptotic cells may cause reduced IFN- γ levels in cultures of antigen-stimulated PBMC. Indeed, neutralization of IL-10 partially restored the IFN- γ production in the presence of apoptotic cells (not shown). Further, injection of apoptotic cells significantly impaired cell-mediated immune responses in mice as measured by delayed-type hypersensitivity reaction (R. E. V. *et al.*, manuscript in preparation).

Conditions causing apoptotic cell death are usually associated with suppression of inflammation and cell-mediated immunity. Moderate ultraviolet irradiation of the skin potentially induces IL-10 expression by immunosuppressive tolerance-inducing CD36⁺ CD11⁺ macrophages^{8,9}. Increased exposure to apoptotic cell material during tumour growth, and many viral infections including HIV, may contribute to the impaired immune response in these conditions. During embryo implantation, uterine epithelial cells surrounding the blastocyst undergo apoptosis¹⁰ and may form an anti-

inflammatory environment by increased IL-10 expression⁷ preventing immunological rejection of the embryo. Also, immunosuppression after transfusion of incompletely leukocyte-depleted autologous and allogeneic blood might be caused by apoptotic leukocytes¹¹.

It should be possible to interfere with the immunosuppressive effects of apoptotic cells for therapeutic benefit, by blocking their interaction with CD36. Agonistic CD36-ligands such as specific antibodies or apoptotic cells may be helpful in controlling graft rejection, inflammation and autoimmunity.

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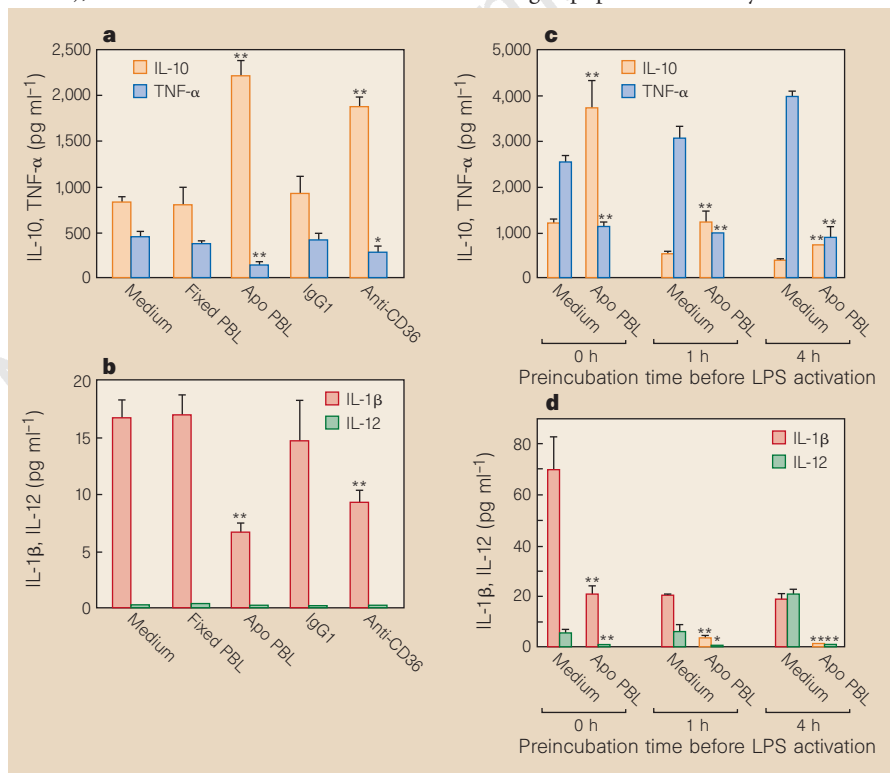


Figure 1 Modulation of cytokine secretion in LPS-activated PBMC and monocytes by apoptotic cells and anti-CD36 antibodies. **a,b**, Concentrations of IL-10, TNF- α , IL-1 and IL-12 in supernatants of PBMC activated for 16 h with LPS in the absence (medium), or in the presence of either freshly prepared paraformaldehyde-fixed autologous PBL (fixed PBL), autologous ultraviolet-irradiated apoptotic PBL (apo PBL), isotype-matched control monoclonal antibodies (IgG1), or CD36-specific antibody FA6-152 (anti-CD36). **c,d**, Influence of the incubation time of monocytes with apoptotic cells before LPS stimulation on the cytokine secretion pattern. Means $\pm$ s.d. of quadruplicate (**a,b**) or triplicate (**c,d**) cultures, representative of at least 3 experiments. Asterisks indicate $P < 0.05$ (*) or $P < 0.01$ (**) in comparison to medium control (Student's *t*-test).

Why parent birds play favourites

Most birds hatch their broods asynchronously, which leads to reduced growth and higher mortality of last-hatched nestlings^{1–3}. Why parent birds confer handicaps on some of their progeny, and advantages on others, remains controversial, although a consensus is emerging that there is no single adaptive explanation^{2,3}. Our studies of red-winged blackbirds provide confirmation of an insurance hypothesis^{4–9} where the marginal offspring created by hatching asynchrony serve as replacements for failed earlier-hatched or 'core' offspring.

Insurance theory predicts that the phenotypic handicap imposed by parents upon marginal offspring is reversible — the mortality of marginal offspring falling when core offspring fail⁸ — and buffers core offspring

from the effects of overcrowding when marginal offspring prove redundant⁹. We conducted field studies on red-winged blackbirds (*Agelaius phoeniceus*) over seven field seasons at Norman, Oklahoma (1991–93) and Winnipeg, Manitoba (1993–96). The clutch size of blackbirds at the two sites ranged from two to five eggs (mode, four). Core and marginal offspring⁶ were defined on the basis of the day of hatching. Brood size at eight days post-hatch was used as an index of fledging success. Site differences in the average mortality of core and marginal chicks were small and non-significant; data on nestling survival were therefore pooled.

We experimentally enlarged or reduced clutches by removing or adding single eggs during egg-laying. In a small number of cases, we enlarged or reduced broods by moving a chick at hatching. Sham control broods were created by reciprocally swapping eggs across nests to control for any potential effects of handling. The mortality of core and marginal chicks in sham control broods was virtually identical to that in unmanipulated broods (Fig. 1), thus the experimental treatment had no discernible effect. To avoid pseudoreplication, the mortality of core or marginal chicks was averaged for each brood, and two-tailed comparisons based on the difference in sample means (D) were performed using approximate randomization¹⁰; 95% confidence intervals were estimated by bootstrapping¹¹.

Mortality for marginal offspring in

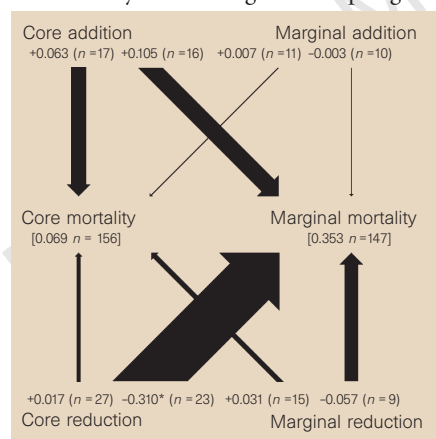


Figure 2 Effects of core and marginal brood reductions and enlargements on core and marginal chick mortality. Numbers in square brackets in the centre represent the average mortality and sample size of core and marginal chicks in unmanipulated broods with no hatching failure, used as a baseline for all comparisons. Arrow width represents the point estimate of the effect of an experimental enlargement or reduction on mortality and associated numbers give the exact difference between unmanipulated broods with no hatching failure and experimental broods. Numbers in parentheses are sample sizes for experimental broods. Asterisks indicate significant ($P < 0.05$) differences between unmanipulated control and experimental broods in approximate randomization tests of differences in means.

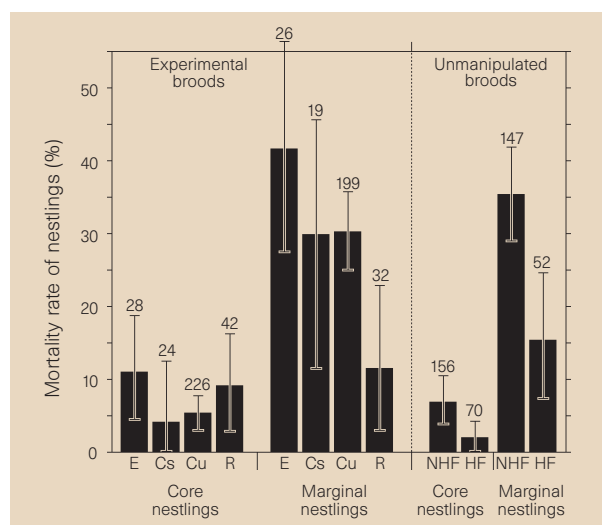


Figure 1 Mortality of core and marginal offspring. E denotes a clutch enlargement; R a clutch reduction; Cs, sham control broods; Cu, unmanipulated control broods; NHF, unmanipulated nests with no hatching failure; HF, unmanipulated broods with hatching failure. Vertical lines represent 95% confidence intervals with sample sizes shown above.

broods without hatching failure was more than five times higher than for core offspring in unmanipulated nests with no hatching failure (Fig. 1). But the phenotypic handicap was reversible, as predicted under insurance theory. Marginal offspring mortality was lower in non-experimental broods with hatching failure than in broods without hatching failure ($D = 0.199$, $P = 0.002$; Fig. 1), and marginal offspring mortality was lower in experimentally reduced broods than that in unmanipulated control broods ($D = 0.187$, $P = 0.012$), sham control broods ($D = 0.184$, $P = 0.068$), or enlarged broods ($D = 0.302$, $P = 0.001$).

As predicted by insurance theory, the phenotypic handicap buffered core offspring from the effects of overcrowding when core failure did not occur. Our experimental enlargements and reductions of the core and marginal brood show that the number of core offspring influenced the mortality of marginal offspring strongly but the reverse was not true. Core brood reductions, in particular, resulted in a sharp reduction of marginal offspring mortality, whereas the number of marginal offspring had virtually no effect on core offspring mortality, even when the marginal brood was experimentally enlarged (Fig. 2). Thus, whether marginal chicks lived or died was rendered inconsequential to the fate of core offspring by the competitive asymmetry imposed by hatching asynchrony.

Hatching asynchrony can serve several functions simultaneously — protection against whole brood loss, uncertain food supplies, or sex-ratio bias — probably reflecting a balance among these^{1–3,7–9}. However, insurance is the only general advantage of hatching asynchrony in birds, being the only viable explanation for obligate brood reduction^{2,4,6,9}. All birds, including facultative brood-reducers, face the risk of early offspring mortality^{5,12}. Parents can protect themselves against this contingency, and enjoy the benefits of insurance, by holding a low-cost replacement in reserve. Hatching

asynchrony creates a caste of low-cost, disposable offspring.

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Correction

In “Tree-ring dating the 1700 Cascadia earthquake” by David K. Yamaguchi *et al.* (**389**, 922–923; 1997) the final paragraph contained some ambiguity. A corrected version follows:

The dates provide a simple test of earthquake size. Suppose that the dates excluded January 1700. In that case, the tsunami in Japan could not represent a Cascadia rupture longer than the 650-km distance between the region of the dated snags and the far (California) end of the subduction zone. Because a 650-km-long rupture at Cascadia would be too small for magnitude 9 (ref. 7), dates excluding January 1700 would strengthen geophysical arguments⁸ against magnitude 9 earthquakes at Cascadia. By instead converging on January 1700, the dates mean that the northwestern United States and adjacent Canada are plausibly subject to earthquakes of magnitude 9.

Watching the sex detective

Alfred C. Kinsey: A Public/Private Life

by James H. Jones

Norton: 1997. Pp. 937. \$39.95, £28

Lesley A. Hall

Apparently the antithesis of its subject's intellectual preference for the analysis of huge samples over in-depth studies of individuals, James H. Jones's 900-odd-page biography (longer than either of the *Kinsey Reports*) is deeply Kinseyan in its relentless accumulation of data.

Biography as social history, 27 years in the making, this detailed study of Alfred Kinsey's origins, boyhood and youth, and his subsequent career as an academic biologist within the university system is valuable in itself. An extended account of Kinsey's work on gall-wasps indicates his stature in this field and how his work related to broader scientific developments. His significance to biology teaching in mid-century America is also established.

But the reason why Kinsey is assumed to merit a biography of this density is, of course, his massive and influential studies of human sexual behaviour, and, however desirable this broader contextualization, the work must stand or fall by its treatment of what made Kinsey a household name.

A question that immediately springs to mind is whether the mass of material serves as prudent wrapping for Jones's sensational revelations about Kinsey's own sex-life? These are startling, even shocking, certainly, but Jones's treatment of Kinsey's autoerotic and homoerotic activities is by no means salacious. Indeed, they are described with almost too much detachment — the attitude of observing an interesting or bizarre specimen may be in harmony with Kinsey's taxonomic science, but sits a little oddly in a biography.

It is perhaps not easy to empathize with Kinsey's taste for extreme auto-masochism, nor with the agenda of sexual liberation, mate-swapping, exhibitionism and voyeurism into which he inducted his colleagues at Indiana. It is no longer possible to treat these manifestations as might have been done in the 1970s, as an uncomplicated and personally transformative throwing over of the barriers of repression.

But need Kinsey be seen as the guru of a sinister cult? He fits into a tradition of writers claiming to work for the reinforcement of marriage (the only strategy in the earlier twentieth century, apart from pathologizing the 'abnormal', permitting discussion of sexuality) whose own sexual and emotional lives were a good deal more baroque than their published recommendations.

In spite of this sexual communitarianism, and indeed the characterization of his work as a subversive Communist plot during the early 1950s, Kinsey stood towards the conservative end of the sex reform spectrum, believing that liberating sexuality and increasing social tolerance for deviance would neither require nor generate major changes in society's institutions.

Jones gives credit to the genuine altruism and desire to serve that underlay, or intertwined with, what, from another angle, can seem a salacious and prurient interest in the sexual activities of others, and to the sympathetic and nonjudgemental attitude that impressed all whom he personally interviewed. If Jones has doubts about the validity of Kinsey's mission of sexual liberation, there is no doubt about the sincerity of his belief in it. Jones does full justice to the complex and ambiguous character of his subject.

Sometimes the evidence is made to bear rather more interpretative weight than it can quite stand: perhaps Kinsey did concentrate initially on the male, and accumulate twice as much material on men, because of his own interest in and identification with them. But consideration of social norms of the period should mediate a too-closely biographical interpretation of this fact: interrogation of women about their sexual lives (especially by a man) was surely even more sensitive than doing so with men; and women were presumably more reluctant to volunteer their experiences.

The resistance to Kinsey's activities from some female faculty members and administrators at Indiana University could well have sprung from conventional prudishness, militating against Kinsey's ability to do for the female what he did for the male. It could, however, equally have represented inarticulate protest against Kinsey's male-orientated assumptions about sexuality. Earlier feminist debates on sexuality had vanished, new ones had not yet been voiced, and no satisfactory discourse was generally available to express dissatisfaction with his implicitly male (though, given his support for the clitoris in the clitoral versus vaginal orgasm debate, not necessarily phallogocentric) model of the sexual.

Kinsey's wife Clara tends to be denied agency in Jones's account: although she was perhaps so under her husband's spell as to go along with his every wish, there are hints from others in their circle that she was something of a 'swinger' by nature. Again, a perhaps rather too narrowly biographical focus ignores the social dimension: surely factors besides husbandly oppression denied Clara an academic career in her own right, while the opportunities for a woman independently to study sexual behaviour, either in person or in theory, as



Alfred Kinsey: complex and ambiguous, and sincere in his mission of sexual liberation.

It is perhaps not easy to empathize with Kinsey's taste for extreme automasochism, nor with the agenda of sexual liberation, mate-swapping, exhibitionism and voyeurism into which he inducted his colleagues at Indiana.

Clara was able to do as her husband's collaborator, were limited in the extreme.

Kinsey has been attacked for the inadequacy of his statistical methods, in particular the biases in representation of the initial sample. But this is hardly the same as lying or making up data (as some groups have claimed of him). Even his opponents seldom denied the actual veracity of his findings, only the wisdom of so publicly communicating them, or the grimly mechanistic view of sex he presented.

Unlike most of his contemporaries in the sex research field, he was not dependent on a criminal or psychiatric population for his evidence, and his work stands head and shoulders above the shoddiness of contemporaneous investigations into the supposed biological (in those days, hormonal) basis of homosexuality. The "Kinsey Scale" may be a simplistic device for calibrating the vagaries of sexual desire, but compared with the usual binary either/or model it was a massive paradigm shift.

Although it is possible to quibble about exact figures, the abiding message of the *Kinsey Reports*, ironically supported by the work of the predecessors whom Kinsey scorned as working from much too small and skewed samples, stands: sexual behaviour in the human, male and female, is far more diverse than the model set up by conventional morality would have us believe. For many they carried the reassuring message "You're not alone". □

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Dogma or debate?

Old Wine, New Flasks: Reflections on Science and Jewish Tradition

By Roald Hoffman and Shira Leibowitz Schmidt

W. H. Freeman: 1997. Pp 362. \$28.95, £17.95

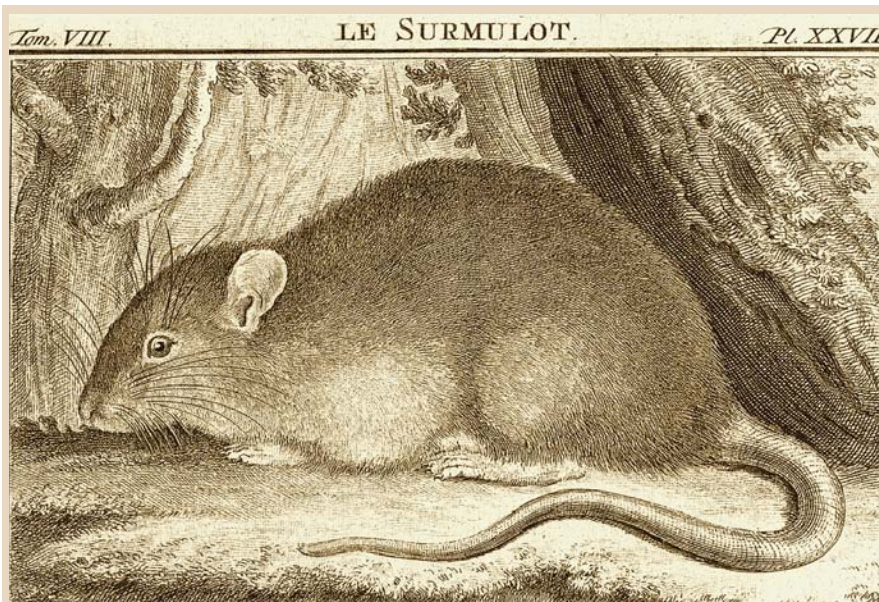
George Klein

The authors — one a Nobel laureate in chemistry, the other a Talmudic scholar and teacher who is the mother of six children — offer the reader "juxtaposed tales of science and Jewish religious tradition, in which scientists, doing experiment and theory, face concerns similar to those of human beings in the Bible, the sages of Talmud, and thoughtful people throughout the ages". There is, they say, "a parallel not only in subject matter, but in the logic applied. There is a set of hypotheses, precedent, a way of thinking There is incredible continuity and clever, clever thought."

This is a well written book. It is witty and erudite, full of the most unexpected associations and contrasts. It alternates between the sublime and the mundane, between deep thought and funny anecdotes while jumping, entertainingly, between different styles, including fictive e-mail exchanges and short theatrical plays. Both authors must be great teachers. Nevertheless, the basic idea of the book, as expressed in the title metaphor, is an absurdity, or, to use a Hungarian proverb, "an iron ring made out of wood".

Even though the Talmudist author is apparently well versed in modern science, her reasoning rests, naturally, on the firm basis of her religious convictions. This includes a more or less outspoken belief in the divine origin of some of the ancient texts. It could hardly be otherwise, as the biblical stories about the creation of the world, the words and miracles of God, and His direct intervention in the lives and doings of His people provide the basis for all Talmudic reasoning. The rest is interpretation and commentary or practical rules.

It cannot be denied that the dynamic intensity of the continuous debates in the *yeshivot* (Jewish Orthodox religious colleges) are reminiscent, at least superficially, of the discussions at some of the best scientific



Rat pack

Eighteenth-century engraving from a drawing by Buffon of "Le Surmulot", the brown rat. The picture is reproduced in *The Rat: A Perverse Miscellany* by Barbara Hodgson (Ten Speed Press, \$15.95), an elegant and entertaining little

paperback devoted to the animal's habits and accomplishments, but most of all to centuries of literary and pictorial evocations (from Hieronymus Bosch to James Bond) that show our deep fascination with this despised rodent.

meetings. Living in the neighbourhood of some religious schools during my working periods in Jerusalem, I could not fail to notice the agitated joy of the traditionally dressed young men, often engaged in lively exchanges, as they walked to school every morning. Except for their garments, they reminded me of some unforgettable post-breakfast walks at Cold Spring Harbor or at a Gordon Conference. But don't be misled. In spite of superficial similarities at the psychological and sociological level, the core substance is totally different.

Not that the habit of argumentation and scholarly competition in the Talmudic tradition may not have contributed to the evolution of scientific discussions. When another Nobel laureate, the great American physicist Isidor Rabi, was asked whether his attendance at a religious school in pre-war Poland had influenced his later scientific development, he answered in the affirmative. But he also pointed out that the most important influence came from his mother. When ten-year-old Isidor came home for lunch, she did not ask whether he did well at school, like most other mothers. She wanted to know whether the boy had asked a good question.

So far so good. But the crux of the matter is that the discussions at the *yeshivot* never question the basic assumptions about God (spelled G-d) and "His" absolute truths.

How do scientists who are also Orthodox Jews handle the contradictions between religion and science within themselves? The legendary and recently deceased Professor Yeshayahu Leibowitz, who happened to be the

father-in-law of the second author, is a particularly interesting case in point. He was a professor of chemistry, but also lectured and wrote on many other subjects, such as neurophysiology, philosophy, literature and numerous Judaistic themes. A meticulously observant Orthodox Jew, Leibowitz had a strictly compartmentalized mind. The religious law had to be kept and not discussed, because it was the law. Philosophy was the realm of sceptical criticism, science was the field of experimentation and discussion. Molecular biology was the greatest scientific advance of the century but it could not explain embryonic development or the workings of the mind, now or in the future. Leibowitz was a Kantian dualist with a watertight partition between non-communicating worlds, each of which had its consistent logic.

The authors of this book do not attempt to pierce this partition, nor do they acknowledge its impenetrability. Rather, they try to joke it away. I can only admire the way in which they use a gamut of scientific explanations for biblical episodes such as Moses' turning bitter waters into sweet by throwing a particular tree into a lake, following direct divine instruction. Having presented a variety of chemical guesses in e-mail form, the authors and some of their real or fictitious discussion partners are finally put in their place by an outstanding biblical scholar, Professor Moshe Greenberg. He is quoted as saying: "Such attempts of scientists to rationalize or naturify Biblical miracles, while undertaken in a spirit of piety, defeat the very purpose of the Bible in reporting them. What place a faith

in miracles has in the belief of moderns is a separate serious issue that no eviscerating rationalisation of Biblical miracle stories can help to determine."

Miracles apart, the book contains valid insights about natural phenomena in the ancient texts. The Talmud, like other sources of old wisdom that have been collected and preserved over many centuries, records innumerable pertinent observations about the world outside and within us. Some of them belong to the realm of chemistry, and their thorough presentation from both sides provides instructive entertainment. Basically, however, they are not very different from other ancient sources of popular wisdom, 'traditional medicine' or even folklore.

Readers with a warm feeling towards the Zionist ideal may welcome the section on *tekhelet*, a blue dye derived from a snail, that has been used in accordance with direct biblical instruction to colour religious garments and instruments of prayer over many centuries and has reemerged in the Israeli flag. But the theatrical conversation between Theodor Herzl and another Zionist leader, David Wolffsohn, at the time of the 1897 Zionist Congress in Basel, quoted as having led to this choice, sounds contrived.

The further projection of the story to the outrageous murder of Yitzhak Rabin, whose coffin was wrapped in the same colours, departs from the main topic of the book in a disturbing way. Its reading induced profound melancholy in the reviewer that was amplified by the ensuing little play. It presents the arguments that have apparently provided the ideological basis for the murder, without an explicit condemnation or comment by the authors. According to these arguments, Rabin was to be regarded as a *rodef*, a pursuer of the Jewish people, a definition that justifies murder according to an ancient law. The fact that the murder was committed at a time when the prime minister had decided, after much agonizing reappraisal, to take the road to peace, is a tragedy that cannot be glossed over by just reporting the differing opinions among the Orthodox themselves on this matter, as the authors do. It is a clear illustration, if any were needed, how strict adherence to a certain interpretation of a religious law may carry the seeds of the ultimate intolerance within itself.

I am reminded, *mutatis mutandis*, of the expulsion of Spinoza — whom Bertrand Russell called "the noblest and most lovable of the great philosophers" — from the Jewish community of Amsterdam in 1656 and the accompanying curse that called on "God's fury and his full malediction" to descend on him. No amount of erudite chemistry, amusing and profound examples of ancient wisdom, no instructive and often funny connections between the old laws or traditions and modern science, can tone down the dangers of religious fundamentalism. Are the authors shying away from the clear condemnation of the

Rabin murder that can be reasonably expected from every moral person, in order to avoid hurting the feelings of religious Jews who share the ultra-orthodox opinion? Do they refrain from addressing some of the incompatibilities between religious judgement and modern science for a similar reason? Perhaps so. But the tactful avoidance of these crucial issues leads to absurdities.

To mention one example, the authors claim there is a "substantive similarity" between the methodology of the Jewish oral law (such as the Talmud) and science, in that they "share a commitment to close analysis and a tradition of citation". The difference is, they say, that Jewish religious discourse always quotes old and even very old statements, whereas science cultivates the new. They add that this "could be the pace of advance; we think it is in part a culture of evaluation of the new. The oedipal urge is stronger in science."

This overlooks — or avoids — the point that everything can be questioned in science but not in religion. There is no scientific paradigm that cannot collapse like a house of cards as soon as new evidence disproves it. Religion adheres to its old texts because of their presumed divine origin. To attribute this difference to the relative strength of an oedipal urge is either another joke or, in the worst case, yet another illustration of the strange blind spots that may affect even the most erudite among us, when we wish to defend our preconceived notions.

The influence of dominating figures is another case in point. The authors emphasize that religious scholars, like scientists, have the right and responsibility to dissent from the views of an authority and that they have a similarly high respect for debate and dialectics. This does not include the right to question the fundamental assumptions, however — as the reviewer, but not the book, hastens to add. Here is the fundamental difference between religion and science. As Niels Bohr pointed out in *Atomic Physics and Human Knowledge* (Wiley, 1958), the goal of science is not to formulate the "universal truth". It has a "more modest but relentless goal: the gradual removal of prejudices". This relentless difference cannot be glossed over by entertaining and instructive similarities between scientific and Talmudic reasoning.

In the final epilogue, the authors list a number of topics that they have avoided, which can be summed up by stating that they have mainly avoided confrontation. What a pity. Taking a less cautious course, they might have succeeded in putting some old wine into new flasks after all, although they would have produced a more controversial brew. As it stands, they have generated only a finely dispersed emulsion of oil in water. Many readers may like it nevertheless. □

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The Mathematical Universe: An Alphabetical Journey Through the Great Proofs, Problems, and Personalities

by William Dunham
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The Origins of Virtue

by Matt Ridley
Penguin, £8.99

"Extremely well written... Ridley does an admirable job of documenting the mutual dependencies implied by Darwin", Frans de Waal in *Nature* 383, 785 (1996)

Painting by numbers

The Invention of Infinity: Mathematics and Art in the Renaissance

by J. V. Field
Oxford University Press: 1997. Pp. 250.
£29.50, \$35

Painting the Heavens: Art and Science in the Age of Galileo

by Eileen Reeves
Princeton University Press: 1997. Pp. 310.
£29.95, £45

Samuel Y. Edgerton

I wish I'd had a mathematics teacher like J. V. Field. I might even have become a mathematician rather than an art historian. Sad but true, we art historians are by nature notoriously maths-shy, even those of us who study the Renaissance.

This is unfortunate because the Renaissance, more than any other cultural period in world history, regarded mathematics as the essence of its art. Mathematicians and artists during the Renaissance were often colleagues, swapping concepts, writing treatises and making images based on each others' expertise. As Eileen Reeves has amply documented, no less a mathematical luminary than Galileo Galilei was a confidant of the artistic celebrities Peter Paul Rubens and Lodovico Cigoli, not only influencing some of their paintings but also being influenced himself by artistic notions that helped him realize some of his most revolutionary astronomical discoveries.

Field's book is indeed a shot of adrenaline in the timid arm of Renaissance art history. Trained as both an art historian and a mathematician, Field plunges right in with a rigorous analysis of the fifteenth-century Italian painter Piero della Francesca's manuscript treatises on mathematics. Although conventional art historians have generally acknowledged Piero's interest in linear perspective, few have dared to work through the artist's heavy-going study on the five regular geometric solids or his 'abacus' book on practical mathematics, much less being able to relate these to his painting procedure. (See also the Art and Science in *Nature* 390, 128; 1997.)

Between the fourteenth and seventeenth centuries, European artists from Italy to the Netherlands became ever more obsessed with the application of Euclidian geometry. Field discusses all this in a clear and jargon-free style frequently spiced with witty asides. But make no mistake, the author panders not one bit to the mathematically disadvantaged. When discussing Tommaso Masaccio's fresco *The Holy Trinity with the Virgin and St John* (c. 1425) in the Santa Maria Novella Church in Florence, for example, she forces the reader to follow her step by step through a complex of possible geometric solutions before admitting that the painter resorted to none of these at all.



Geometrical puzzle: Masaccio's *Trinity* (c. 1425).

As Field goes on to demonstrate, this obsession reached its peak in the sixteenth century when professional mathematicians such as Federico Commandino and Johannes Kepler appropriated artists' linear perspective for application to their own advancing research on conics and the topology of solids. The invention and perfection of printing, moreover, encouraged a plethora of illustrated treatises on linear perspective written also by professional mathematicians and directed back again to artists. The culmination of this rich interchange, as Field concludes, was the seventeenth-century invention of a whole new mathematical science called descriptive geometry by Girard Desargues, again owing to Renaissance painters' practice.

Perhaps no scientific thinker at the turn of the seventeenth century paid closer attention to this expanding application of perspective theory than Galileo. Reeves's elegantly written and exhaustively documented book reveals just how profoundly the great astronomer was involved in the artistic milieu of his native Florence during the first decades of the 1600s. Indeed, Galileo, because of his familiarity with the Renaissance artistic technique of chiaroscuro (light, shade and shadow rendering), could immediately comprehend the importance of two of his most astounding telescopic observations, the scattered shadows on the lunar surface and earthshine, the dim glow on the still dark portion of the waxing or waning Moon caused by light reflected from Earth.

Reeves goes into the religious implications of these discoveries in great detail, even scrutinizing several paintings by Galileo's friend

Cigoli for evidence that the artist was actually justifying Galileo's discoveries by trying to reconcile them to traditional Roman Catholic pictorial iconography.

Cigoli's most famous defence of his friend on this score was the fresco he painted in the cupola of the Pauline Chapel in the Basilica of Santa Maria Maggiore in Rome in 1610–12, supposedly depicting the then popular subject of the Immaculate Conception of the Virgin Mary. According to accepted convention, Mary should be shown standing aloft on a perfectly spherical alabastrine Moon symbolizing her utter purity.

It was this ancient and orthodox notion, of course, that put the Church at odds with Galileo. The Moon, the first of the heavenly planets separating God's realm from the mundane world of mortals, had thus to be, according to sacred doctrine, composed only of perfect ethereal substance as pure as the Virgin herself. What Galileo saw through his telescope, however, quite contradicted this old dogma.

In the face of his friend's evidence, Cigoli made a remarkably courageous decision. The Moon he chose to paint beneath the Virgin's feet displayed the same mountains and craters that Galileo had observed, suggesting that the Moon was hardly ethereal but rather composed of form and matter just like Earth. To this day, the Roman Catholic Church has sidestepped the contradiction apparent in Cigoli's fresco. The painting is never called the *Immaculate Conception*, but simply and uncontroversially *Madonna with Apostles*.

This story has been told before, but Reeves offers much rich new detail including a plausible conspiracy theory that would have Cigoli's nephew secretly censoring his uncle's unpublished perspective treatise of any reference to Galileo, lest his own family name be tarnished by the latter's 'crime'.

I do have one quibble with Reeves's excellent book, however. Nowhere does she acknowledge that Galileo himself was a competent artist in the Florentine Renaissance tradition, that he did not need to consult written treatises of others to comprehend earthshine on the Moon. Any well-trained cinquecento draughtsman, as Galileo certainly was, would have known how to recognize and render reflected light.

Proof is apparent in the wash drawings of the phases of the Moon that Galileo himself rendered in preparation for the engravings published in his 1610 *Sidereus nuncius*. These renderings are now preserved in the Biblioteca Nazionale in Florence, and clearly demonstrate Galileo's own natural proficiency in "painting the heavens". Too bad that Reeves in her book did not reproduce any of these, the most Galilean of all artworks inspired by the astronomer's telescope. □

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Geological record and reconstruction of the late Pliocene impact of the Eltanin asteroid in the Southern Ocean

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In 1995, an expedition on board the research vessel FS *Polarstern* explored the impact site of the Eltanin asteroid in the Southern Ocean, the only known asteroid impact into a deep ocean basin. Analyses of the geological record of the impact region place the event in the late Pliocene (~2.15 Myr) and constrain the size of the asteroid to be > 1 km. The explosive force inferred for this event places it at the threshold of impacts believed to have global consequences, and its study should therefore provide a baseline for the reconstruction and modelling of similar events, which are common on geological timescales.

Of the ~140 known terrestrial impacts¹, the late Pliocene impact of the Eltanin asteroid into the Bellingshausen Sea (eastern Pacific sector of the Southern Ocean) is the only example of an impact on the 60% of the Earth's surface covered by deep ocean basins. Evidence of this impact was first discovered as an iridium (Ir) anomaly in 1981 (ref. 2) in a sediment core collected in the mid-1960s by the USNS *Eltanin*. Subsequent studies³ showed that meteoritic ejecta are present in three sediment cores separated by 500 km and that the Ir is largely concentrated in vesicular debris that formed by melting of the asteroid as it impacted the ocean. The ejecta contain meteorite fragments of a basaltic achondrite⁴ that have been named the Eltanin meteorite (an anomalous mesosiderite), and rare spherules containing Ni-rich magnesioferrite spinel⁵, a component also found in Cretaceous/Tertiary (K/T) boundary ejecta⁶.

Here we present the results of a more comprehensive exploration of the Eltanin impact area, combining bathymetric, seismic and marine-geological studies, accomplished during the 1995 expedition ANT-XII/4 of the FS *Polarstern*. The surveyed area is in the vicinity of the location of Eltanin Core E13-4 (57°47.2' S, 90°47.6' W, 4,700 m depth), where the highest concentrations of impact debris were encountered³. We found evidence of erosion and redeposition of sediments of various ages covering a time span of 50 Myr. We describe an allochthonous impact-related sedimentary succession resting with erosional contact on autochthonous pre-impact sediments. This documents the impact-triggered resuspension and mass transport of sediments from the sea floor and the subsequent decrease in turbulent energy during redeposition of this reworked material. The lower part of the sequence is made up of a 'chaotic', unsorted intrabasinal breccia that includes sediment clasts of different sizes, lithologies and ages overlain by a laminated and graded foraminiferal sand. The uppermost part of the sequence consists of biogenic oozes of various ages that were sedimented from a cloud of sediment dispersed in the water column. Meteoritic ejecta, which were probably airborne and settled through the 5 km water column, began to accumulate on the ocean floor 4 hours after

the impact. The impact might be responsible for producing a seismically transparent sediment layer, typically 20–40 m thick and extending hundreds of kilometres across the ocean floor. Although there is evidence for disturbance and removal of large volumes of sediments our survey did not find evidence that a crater was excavated on the ocean floor.

We estimate that the size of the impacting asteroid was in the range of 1–4 km with an explosive force in the range of 10^5 – 10^7 Mt TNT explosive. Although an impact of this size would have excavated a 15–40-km diameter crater on a continent⁷ it is unlikely to penetrate the crust of the deep-ocean basins. In contrast to continental impacts, the Eltanin impact might have caused devastating megatsunamis along many coastlines and might have had an effect on the climate by the ejection of large volumes of water and salts that would be deposited in the upper atmosphere. The ~2.15-Myr age of the impact precedes a period of intensified cooling. Curious phenomena reported for the Pliocene might be related to this impact, including unusual bonebeds in Peru, marine Pliocene sediments covering coastal Antarctic areas, and marine microfossils in the Transantarctic Mountains.

Survey in the impact region

The survey area is on the Pacific–Aluk portion of the Antarctic plate⁸, where basement ages are ~60 Myr (Fig. 1a). Topography mapped by Hydrosweep swath-bathymetry revealed an irregular seamount system, the San Martin seamounts, with minimum water depths of ~2,500 m. The surrounding rugged deep-sea floor has depths of ~5,000 m (Fig. 1b).

In the abyssal portions of the survey area, a high-resolution seismic survey with a Parasound echosounding system revealed a sequence of well-stratified reflectors typically 20–30 m thick, underlain by a seismically transparent zone. The base of the Eltanin–Polarstern Transparent Zone (EPTZ) is a strong reflector, which prevents deeper penetration of the acoustic signals in most areas. In some places, such as north of the seamount, the EPTZ increases to more than 60 m, filling in an irregular topographic structure, where

the strong reflector probably marks basement (Fig. 2). In the areas east of the seamounts the EPTZ displays a more uniform thickness ranging between 20 and 40 m, following the bottom topography. The EPTZ was not discerned on the seamounts, where rough topography disturbed the echosounding signal. Seismically transparent layers can represent sequences of stratified sediments with almost zero acoustic impedance contrasts at their layer boundaries or a zone of chaotically deposited sedimentary material. Thus the EPTZ was suspected to be related to deposits disturbed by the impact.

At most localities, the EPTZ was beyond the reach of our 20-m-long piston coring device because of the greater thickness of the overlying acoustically stratified sequence. However, we successfully

penetrated the EPTZ at eight localities where we observed that the seismically stratified sediments and the EPTZ are thinning towards the edges of topographic highs and hence we were able to recover impact deposits. Three cores with complete sections provide a depth profile from Core PS2709-1 near the top of the seamount ($57^{\circ}46.8' \text{ S}$, $90^{\circ}59.9' \text{ W}$; 2,707 m depth), to PS2708-1 at intermediate water depths ($57^{\circ}35.4' \text{ S}$, $91^{\circ}13.2' \text{ W}$; 3,965 m), and to PS2704-1 in the abyssal basin north of the seamount ($57^{\circ}23.4' \text{ S}$, $90^{\circ}48.4' \text{ W}$; 4,961 m; Fig. 1b). In two cores (PS2714-1, PS2705-1) we recovered only the uppermost portion of the impact deposit and in three others (PS2702-1, PS2706-1, PS2710-1), significant parts of the section seem to be missing because of an erosional event that affected sediments around the seamounts in the early Pleistocene.

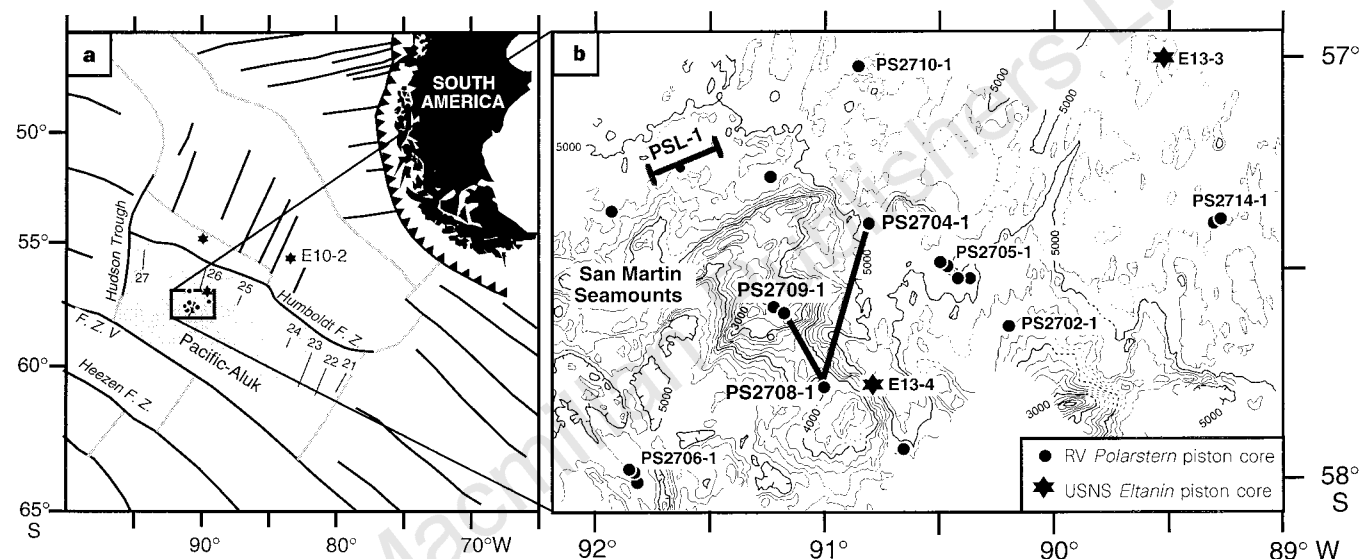


Figure 1 Schematic representation of the Bellingshausen Sea tectonic setting showing major fracture and subduction zones; the Pacific-Aluk portion of the Antarctic plate is shaded and shows magnetic lineations in accordance with ref. 8 (a). Box on Pacific-Aluk indicates area with extensive bathymetric, seismic and sediment coring survey in the vicinity of San Martin seamounts (b). Bathymetric

map (200-m contour interval) of area affected by the asteroid impact shows the location of core transect PS2709-1-PS2704-1 (Fig. 3), and other Polarstern and Eltanin cores discussed in the text. PSL-1 indicates the location of the Parasound echosounding profile in Fig. 2.

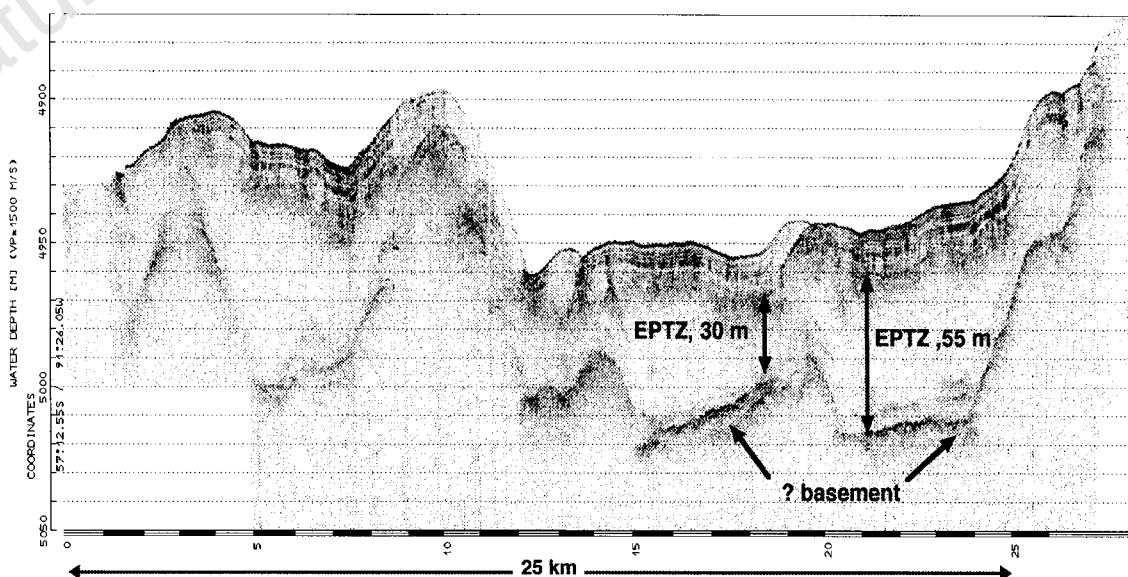


Figure 2 Parasound echosounding of the sediments along the transect PSL-1 to the north of the seamounts (Fig. 1b). Up to 60 m of seismically transparent sediments, representing the Eltanin-Polarstern Transparent Zone (EPTZ) underlie the well-stratified late Pliocene to recent sediments and fill a rough topography

that might be basement. The EPTZ might be largely sediments ripped up and redeposited by the impact. The Parasound system typically allows seismic penetration of the upper 100-300 m of sediment.

We recognize five sedimentary units (SU). The lowermost SU V consists of autochthonous, undisturbed sediments. In PS2708-1 the lowermost 163 cm are SU V consisting of a calcareous nannofossil ooze assigned to the middle Eocene (NP16, ~43–41 Myr) (Fig. 3). The clays in the ooze bear >90% well-crystallized smectite, with associated clinoptilolite and goethite. In PS2704-1, a homogeneous sequence of clay (~80% smectite) with frequently changing magnetic polarities at mostly steep inclinations occurs below 1,411 cm. However, traces of diatoms with a first occurrence datum in the late Pliocene predating the impact event (*Fragilariopsis kerguelensis*) cause us to suspect that this sequence might be partly disturbed and more appropriately belongs in SU IV, except the section below 1,733 cm, where clays are almost pure smectite (99%) like that in the Eocene section of PS2708-1 (Fig. 4).

The impact-related deposit begins with SU IV, an allochthonous, chaotic mixture of sedimentary clasts as large as 50 cm, with different lithologies and ages (Figs 3, 5a). At the top of the seamount (PS2709-1) SU IV is composed of clasts of middle Eocene to Oligocene, middle Miocene and Pliocene calcareous nannofossil and foraminiferal oozes (~90% CaCO₃) with minor admixtures of Pliocene diatoms and radiolarians (Fig. 4). In PS2708-1, SU IV contains manganese nodules, and a fragment of a ~15-cm-thick manganese crust interbedded with soft sediment clasts of barren clays, middle Eocene calcareous muds and Pliocene diatom oozes

and muds. In PS2704-1 SU IV has two distinct subunits (Fig. 3). The lower, SU IVB, contains sediment clasts up to 15 cm in diameter that are composed of clays with only traces of poorly preserved diatoms, probably Pliocene in age. SU IVA is characterized by smaller clasts (0.5–2 cm) embedded in a sandy matrix and contains up to 40% carbonate composed of Eocene to Oligocene foraminifera and nannofossils besides rare diatoms that range in age around the Oligocene/Miocene boundary, in the middle–late Miocene and the Pliocene.

SU III is a laminated, well-sorted and graded sand (Figs 3, 5b). In PS2709-1 it consists predominantly of Pliocene foraminifera with admixed Palaeogene and Miocene calcareous nannofossils. In PS2708-1 and PS2704-1, SU III is a mixture of biosiliceous and calcareous sediments including Palaeogene and Neogene microfossils (Fig. 4), as well as a few basaltic rock fragments (PS2704-1).

SU II consists mainly of fine-grained sediment (Fig. 3). Whereas in PS2708-1 this unit is ~115 cm thick and unbioturbated, in PS2709-1 and PS2704-1 it is much thinner and significantly disturbed by bioturbation. In the shallow sites (PS2709-1, PS2708-1) above the carbonate compensation depth (CCD) SU II is characterized by the occurrence of displaced Palaeogene calcareous microfossils, mainly Eocene in age (Figs 4, 6). The deep PS2704-1 contains reworked Neogene and Oligocene diatoms and clinoptilolite, most probably originating from Palaeogene clays (Fig. 4).

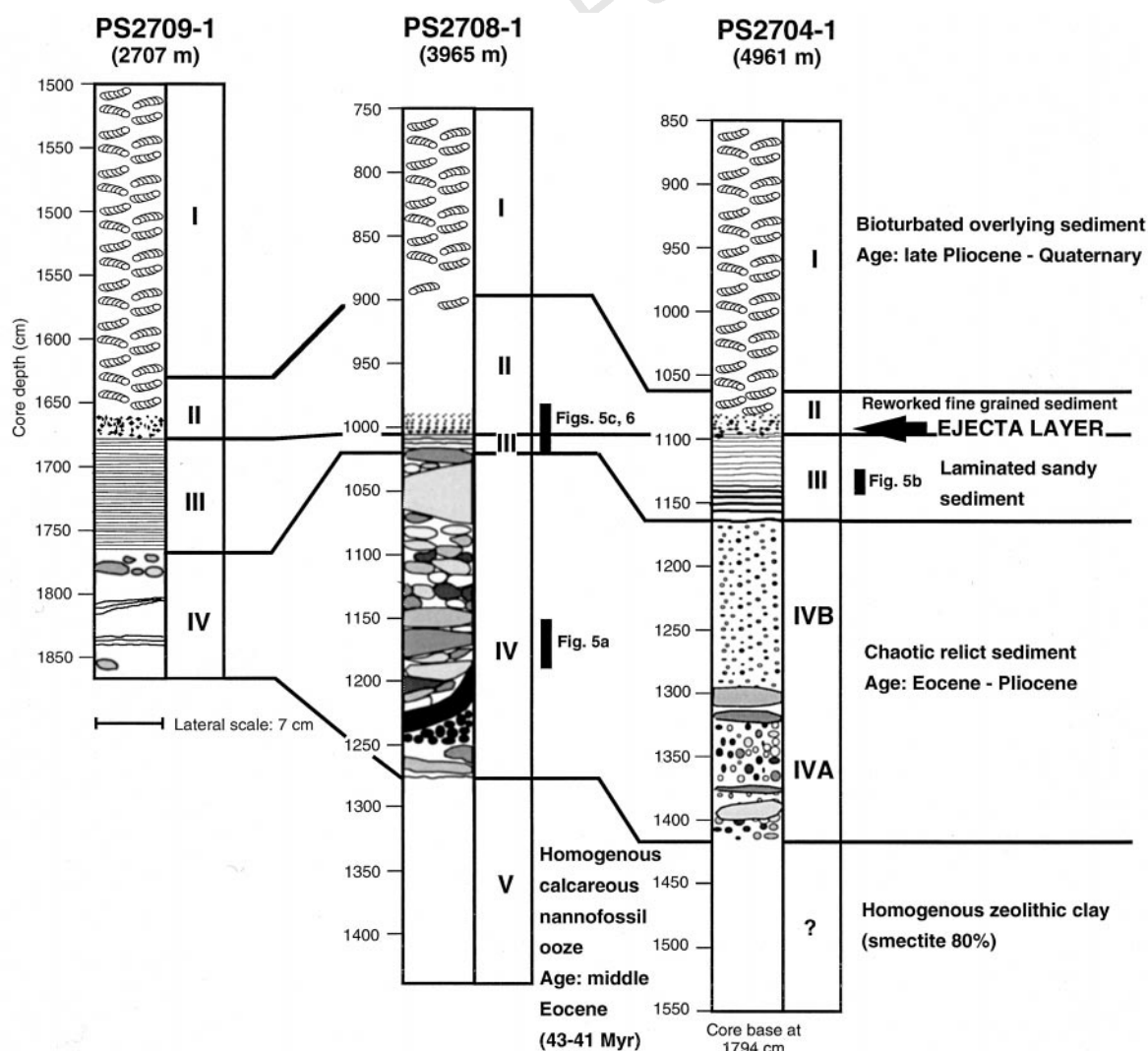


Figure 3 Piston cores PS-27091-1, PS2708-1 and PS2704-1 provide a depth transect from the top of the San Martin seamounts to the abyssal floor in the north (Fig. 1b). Five sedimentary units are recognized. SU II, III, and IV were deposited in

the brief interval after the impact. Core logs include information on sediment structure only.

Meteoritic ejecta were found within the uppermost portion of SU III and throughout SU II. Maximum concentrations making up to 20% of the total sediment occur in the lowermost 10–20 cm of the ejecta-bearing interval. The meteoritic particles reveal an upward decrease in grain size overprinting the grain size distribution of the

non-meteoritic material (Fig. 6). The ejecta consist mainly of vesicular impact melt, but few per cent of the ejecta are unmelted meteorite fragments, and trace amounts of spinel-bearing spherules have been observed. We have separated 7.9 g of coarse impact debris. Of this, 0.42 g are unmelted meteorite particles, making the

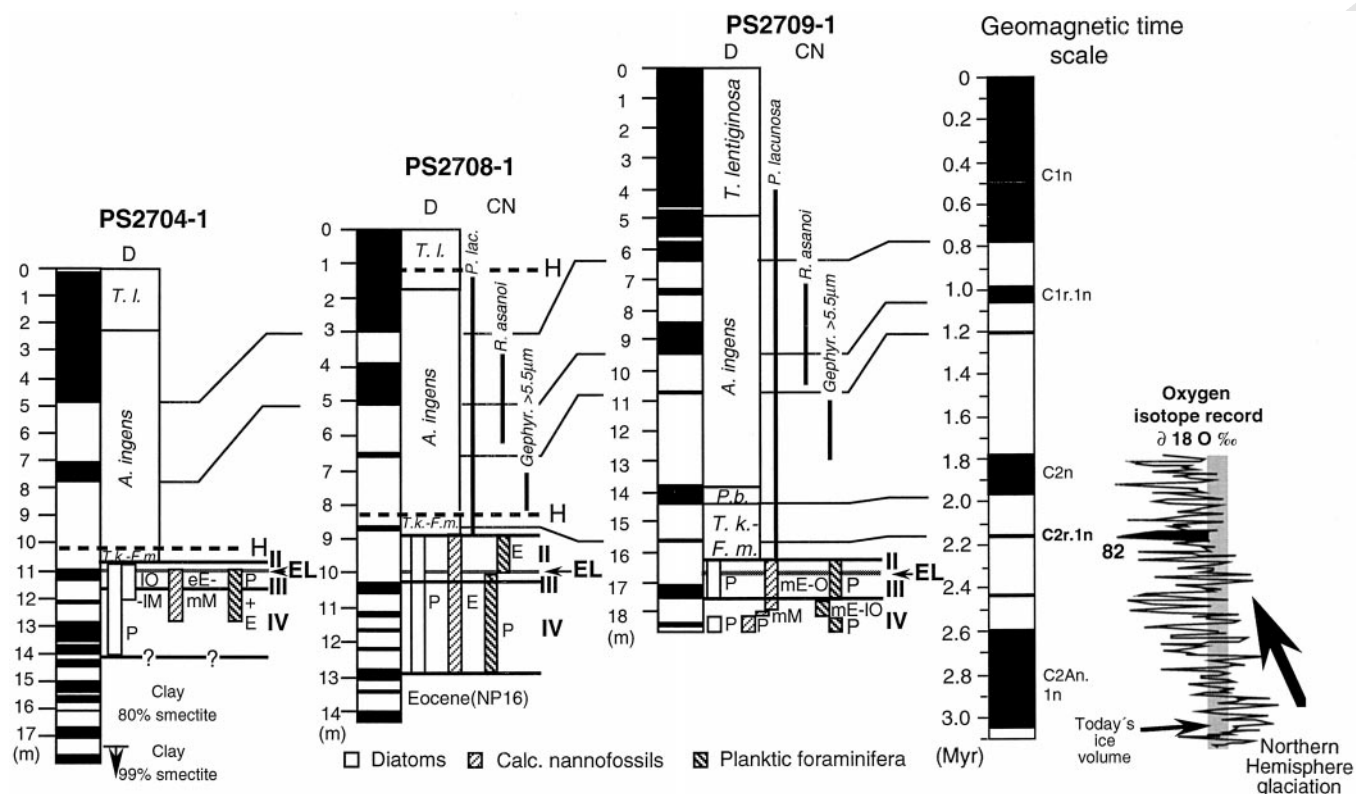


Figure 4 Age determination of the impact event and reworked impact deposits based on combined magnetostratigraphy and biostratigraphy of PS2704-1, PS2708-1 and PS2709-1. Impact-related SU IV, III and II and ejecta layer (EL) are marked. The youngest impact-related SU II is close to the Réunion geomagnetic event (C2r.1n). The impact event thus closely predates C2r.1n, and is close to isotope glacial event 82 (ref. 32). Bars on the impact-related interval indicate the schematically stratigraphic ages of diatoms, calcareous nannofossils and planktic foraminifera predominantly recovered in SU IV, III and II (eE, early Eocene; mE, middle Eocene; IO, late Oligocene; mM, middle Miocene; IM, late Miocene; P, Pliocene). Latest Pliocene to early Pleistocene sediments are omitted by a hiatus

(H) in PS2704-1 and PS2708-1. Magnetic data are correlated with the standard geomagnetic polarity timescale¹⁰. Stable characteristic remnant magnetization directions were determined from detailed alternating field demagnetization up to 80 mT strength. Polarities were determined directly from the sign of the steep inclinations. Biostratigraphy by diatom (D) zonation⁹ (zonal names: *T. l.*, *Thalassiosira lentiginosa*; *P. b.*, *Proboscia barboi*; *T. k-F. m.*, *Thalassiosira kolbei-Fragilariopsis matuyamae*) and calcareous nannofossil (CN) ranges^{12,33} and zonation³⁴. Determination of Eocene NP16 Zone (PS2708-1) is based on the presence of taxa such as *Reticulofenestra umbilica* and *Chiasmolithus solitus* and dated following ref. 35.

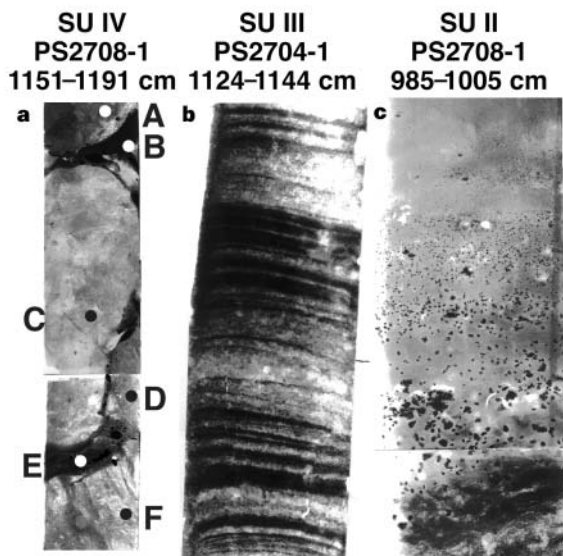


Figure 5 Details of the three sedimentary units formed by the impact illustrated by X-ray radiographs (compare Fig. 3). **a**, SU IV contains ripped up sediment clasts. In the section illustrated, six distinct sediments can be distinguished (A, diatom mud, age ~late Gauss Chron; B, calcareous nannofossil ooze, age ~mid-Eocene; C, diatom ooze, age ~mid-Pliocene; D, diatom mud, age early late Pliocene; E, mixed calcareous Eocene biosiliceous Pliocene; F, diatom ooze, age ~time of impact). **b**, SU III is a laminated sand. In PS2704-1, where it is best developed, it contains carbonates (30–60%) that must have been transported from the seamount ~40 km away. Interval 1,124–1,144 cm contains Palaeogene (~80%) and Pliocene (~20%) planktic foraminifera and late Oligocene–mid-Miocene and Pliocene diatoms. **c**, SU II contains fine-grained sediments that settled from a cloud in the water column consisting of Eocene calcareous nannofossils and foraminifera (Fig. 6) and predominantly mid-Pliocene diatoms. Graded meteorite ejecta can be seen as coarse grains starting at the boundary between SU III and SU II in PS2708-1 at ~1,003 cm depth. Note that the section for SU IV (**a**) is at 50% of the scale for the other two.

unmelted fraction of the meteoritic ejecta 5%. Most of the ejecta mass is in particles from 0.5 to 5 mm in size. The largest pieces have been found in the top of SU III in PS2704-1, including an unmelted meteorite 1.5 cm in length at 1,097 cm, and a 9 mm vesicular particle at 1,096 cm. Although stratification is not evident in the host sediment of SU II, the ejecta are concentrated at different layers in PS2708-1, where the lack of bioturbation allowed the best preservation of the fallout interval (Fig. 5c). This layering is seen to influence Ir and chromium (Cr) concentrations (Fig. 6). Similar, but less well-defined, layering is also present at PS2704-1.

Overlying the impact deposits, SU I is an undisturbed and bioturbated sequence of late Pliocene to Quaternary biogenic sediments.

Analyses of combined magnetostratigraphic and biostratigraphic data, including diatom and calcareous nannofossil stratigraphies, place the base of SU I, and thus the impact age, into the *Thalassiosira kolbei*–*Fragilariopsis matuyamae* diatom zone⁹ directly below the magnetostratigraphic Réunion Event (C2r.1n). This event is in the lower Matuyama Chron and has been assigned an age of 2.15–2.14 Myr (ref. 10) (Fig. 4). Whereas in PS2709-1 SU I has been deposited continuously at sedimentation rates of 0.5–0.9 cm kyr⁻¹, the sediment sequences in PS2708-1 and PS2704-1 are disturbed by a hiatus that omits part of the middle Matuyama Chron (Fig. 4).

The post-impact erosional event might be responsible for the lack of SU II and III, including meteorite ejecta, in such cores as PS2702-1, PS2706-1 and PS2710-1.

We resampled Eltanin cores E13-3, E13-4 and E10-2, all of which are known to contain ejecta³. These cores are in incomplete state and dried out 30 years after their collection. On the basis of available core descriptions¹¹ and our analyses we suggest that they include sediment sequences similar to those documented in the Polarstern cores. In E13-4, recovered close to the seamounts (Fig. 1b), sediments below the ejecta horizon (1,298 cm depth), formerly interpreted as a continuous Eocene deposit¹², seem to be the chaotic SU IV. The sediments are a mixture of barren zeolitic clays rich in well-crystallized smectite (>95%) and Eocene calcareous nannofossil ooze. We also got one sample at 1,305 cm that contains Pliocene diatoms stratigraphically below an early Eocene nannofossil ooze at 1,303 cm. In E13-3 and E10-2, ~100 km and 530 km to the northeast of the San Martín seamounts, respectively (Fig. 1), sediments below the ejecta might also be disturbed by the impact. This is indicated by trace occurrences of diatoms that range in age from early late Pliocene to Quaternary and also in the Miocene, intervals dominated by well-crystallized smectite (E10-2, 750–948 cm) and descriptions of sedimentary structures as ‘lumps and blebs’¹¹.

PS2708-1 (core section 850–1300 cm)

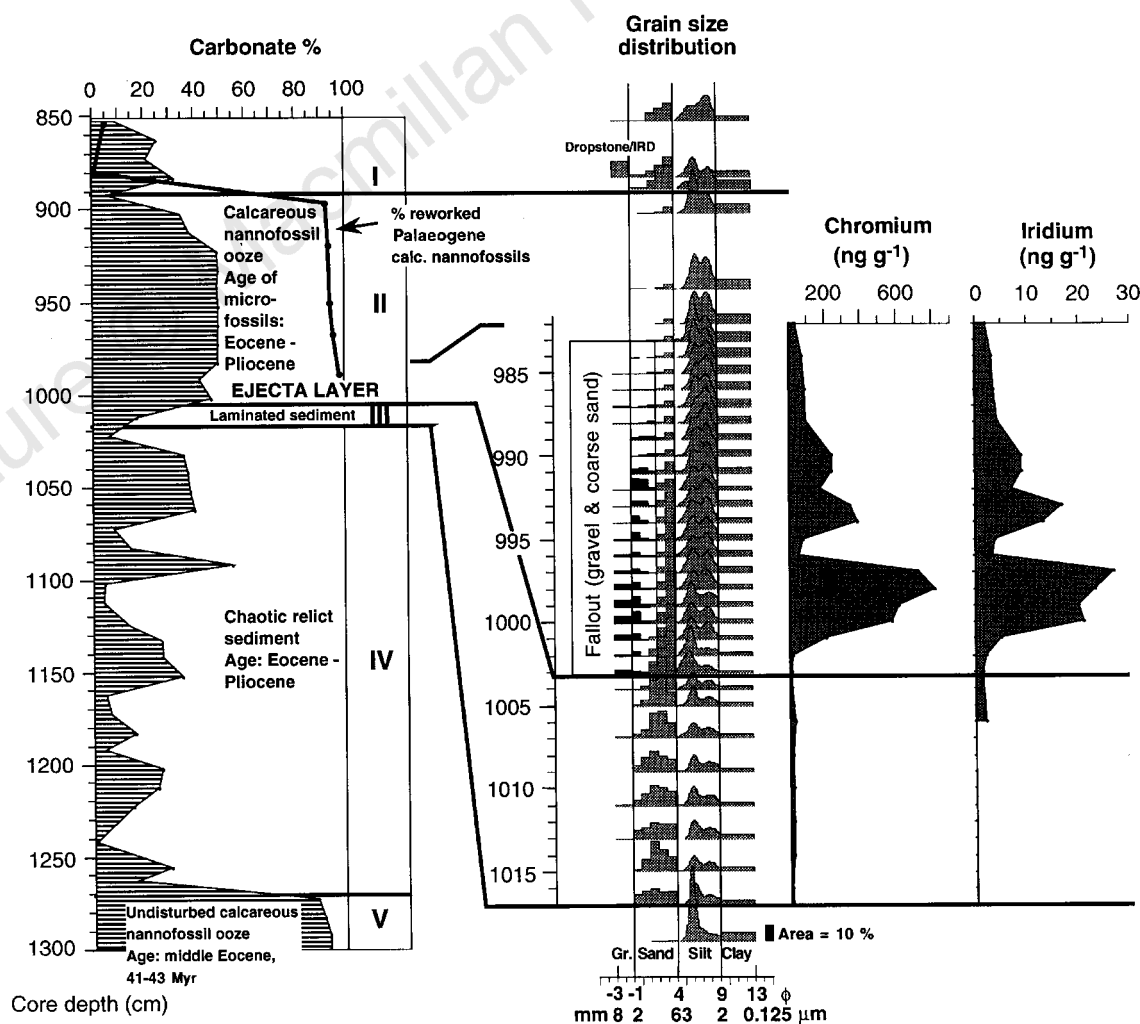


Figure 6 Core PS2708-1 carbonate content in impact-related sediment units, and grain size distribution in an expanded section of the laminated SU III and the ejecta-rich sediments of SU II (Fig. 3). Grain sizes show gradation of sand sizes in the laminated SU III. A sharp increase in gravel and coarse sand components is at

1,003 cm, where the ejecta debris is first observed at the base of SU II. Peaks in the measured concentrations of Cr and Ir coincide with the graded layers of high ejecta concentrations that can be observed in X-ray photographs (Fig. 5c).

Depositional model

We interpret our sediment cores as recording a sequence of processes involving sediment mass erosion and redeposition at decreasing energy level, accompanied by fallout of impact debris through air and water. These processes occurred over a period of at least several hours after the impact event. The impact ripped up and redeposited sediments on the San Martin seamounts and in the surrounding abyssal basin. Some of the displaced sediment has been redeposited rapidly as large fragments, commonly 10–50 cm in size on and in the vicinity of the seamounts (SU IV; Fig. 5a). This involved significant lateral and vertical transport, since fragments of clay-rich sediment typical of the abyssal basin were found above the CCD in PS2708-1, ~1 km above the present abyssal sea floor. In turn, carbonate-rich clasts and calcareous microfossils were deposited out in the basin below the CCD as recorded in PS2704-1, ~40 km northeast of the seamount crest.

The laminated sediments of SU III (Fig. 5b) were deposited from a turbid flow. The presence of SU III in the abyssal areas (PS2704-1) as well as on the top of the seamount (PS2709-1) indicates that there was both downslope gravitational sediment transport into the abyssal basin and rapid sedimentation of well-sorted sand-sized particles (for example, foraminifera) from sediments stirred up into higher levels of the water column. We can estimate the duration of this event in the abyssal basin, by using the settling velocity of the 1.5-cm meteorite (measured at 37 cm s^{-1}) recovered from the top of SU III at PS2704-1. On the assumption that this rock settled through the water column (~5,000 m) to reach the abyssal floor, deposition of SU III did not conclude at site PS2704-1 until 4 hours after the impact.

SU II records the settling of fine-grained particles through the water column including a cloud of resuspended bottom sediment, and meteoritic ejecta that were most probably airborne. Some of the non-meteoritic sediment might also be ejecta, but we lack criteria to distinguish this. The observation of concentrations of the meteoritic ejecta in different horizons, documented in X-ray radiographs (Fig. 5c) and Ir and Cr distributions in PS2708-1 (Fig. 6), indicates that currents were still active during the early phases of sedimentation of SU II. The transition from SU III to SU II might reflect a gradual transition to less turbulent conditions, rather than a boundary between two distinct events. Deposition of SU II probably lasted for several hours to days after the impact.

At sites further from the seamounts, our information is less detailed but there is evidence of disturbance over a broad region. At PS2714-2 ($57^{\circ} 22.7' \text{ S}$, $89^{\circ} 16.1' \text{ W}$; 5,178 m depth; Fig. 1b), ~100 km east, we recovered sediment from the top of SU II showing a mixture of late and middle Pliocene diatoms. At the Eltanin cores, E13-3 and E10-2 mixed microfossil assemblages and clays have been identified below the impact ejecta, indicating that sediment disturbance and the sediment cloud extended at least 500 km to the east of the seamounts. The large areal extent of the EPTZ also indicates widespread disturbance. These acoustically transparent sediments are believed to be the chaotically disturbed unit SU IV. Because we recovered our cores where echosounding indicated that seismically stratified and transparent sediments were thinning, we did not recover the complete disturbed section and we assume that impact units SU II, III and IV in our cores are thinner than typical.

Multichannel seismic data from a west–east profile across the survey area show that in some areas of the abyssal basin sediments are 200–300 m thick, but near the seamounts there are regions where sediment cover is virtually absent. It is possible that the Eltanin impact ejected large volumes of sediment out of our survey area, and that all that might remain is the larger rubble that fills in the exposed topography of the ocean crust.

We know that sediments as old as early Eocene (~50 Myr in E13-4, PS2704-1) were affected by the impact. In fact, early to late Eocene and middle Pliocene sediments are abundant in our samples from SU II–IV. Significant occurrences of diatoms ranging from late

Oligocene to early Miocene (PS2704-1) and rarer findings of microfossils characteristic of the middle and late Miocene indicate that such sediments were also affected by the impact (Fig. 4). We know that Miocene sediments exist in the area because ~3 m of late-middle to late Miocene diatom oozes were recovered at PS2706-1 ($57^{\circ} 58.0' \text{ S}$, $91^{\circ} 51.5' \text{ W}$), ~55 km south of the San Martin seamounts (Fig. 1b).

Sediment reworking is also evident from clay mineralogy. Clay mineral assemblages of the impact-related sediments show compositions intermediate between those characteristic of pre-Miocene altered sediments and younger sediments, indicating mixing of clay minerals from pre-impact strata of various ages. Clay mineral assemblages consisting of almost pure well-crystallized smectite in association with zeolites, as recorded in Eocene clays of PS2708-1 (SU V) and at the base of PS2704-1 (Fig. 4), are consistent with authigenic formation by submarine diagenesis (hydrothermal alteration) and are also known from other parts of the Bellingshausen Sea in sediments older than late Miocene¹³, including Core PS2664-1 recovered ~400 km north of the San Martin seamounts. Sediments younger than the late Miocene differ markedly, consisting of smectite that is only moderately crystallized, and significant amounts of terrigenous illite and chlorite¹³, as also recorded in the late Pliocene to Quaternary sequences of SU I.

Reconstructing the impact event

Previous estimates of the size of the Eltanin asteroid range from 0.5 to 2 km diameter³. The lower estimate was based on a model of the distribution of ejecta that we now find to be too conservative, as it neglected the possibility that a large fraction of the projectile might have been vaporized and distributed globally. High concentrations of ejecta were deposited throughout our survey area. Current estimates of ejecta deposited at sites PS2704-1, PS2708-1 and PS2709-1 are ~500, 830 and 670 mg cm^{-2} , respectively. These compare well with debris estimates of 790 and 187 mg cm^{-2} at E13-4 and E13-3, respectively³. Considering the large area of disturbance (including the deposition of displaced sediment as much as 500 km from the seamounts and the apparent ejection of large volumes of sediment) and the probability that most of the asteroid was ejected far from the impact region, we propose a more reasonable lower limit on the asteroid diameter of 1 km. The 2 km upper limit was constrained by models indicating that during an oceanic impact an asteroid would penetrate the water to depths several times its diameter¹⁴. The latter constraint was necessary because there is no evidence from the impact melt that the projectile mixed with silicate target material⁴. However, recent models indicate that the penetration of the asteroid (its stagnation depth) should be on the order of the projectile diameter⁷, not several times greater. For typical impact angles of 45° , projectiles as large as 3 or 4 km should avoid mixing with the ocean crust. Thus the upper-limit estimate on the Eltanin asteroid should be revised to ~4 km.

At typical asteroid velocities (~20 km s^{-1}), projectiles in the size range of 1–4 km have explosive energies in the range of 10^5 – 10^7 Mt TNT. These energies are at the threshold of those considered necessary to cause global devastation^{15,16}. All impactors in this range will generate devastating megatsunamis. At 1 km the Eltanin asteroid would have generated tsunamis on the order of 20–40 m over deep water approaching South America and Antarctica, which might have reached runup heights on the continental margins 10–25 times higher¹⁷. Deep-water waves 5–10 m high would have extended into the North Pacific and around the Southern Ocean. Evidence of these megatsunamis should be present in sediments throughout the Pacific and Southern Ocean shores. We note geological anomalies that could be related. On the Peruvian coast, near Pisco, an unusual and unexplained mixture of complete marine and terrestrial mammal skeletons was found in coastal marine sediments that could be late Pliocene in age¹⁸. In coastal east Antarctica (Larseman Hills, Windmill Island, Indian sector)

late Pliocene deposits consisting of marine diatomaceous shallow water sediments and only a few decimetres thick are located several tens of metres above recent sea level¹⁹. These are probably allochthonous and might have been caused by up-shore transport of shelf sediments by a tsunami.

The Eltanin impact was certainly large enough for global distribution of ballistic ejecta¹⁶. The ejecta would consist mainly of water from the oceanic target, but would include significant amounts of asteroidal material, salts and probably marine sediments. Deposition of water and salts into the upper atmosphere, and formation of NO caused by the ionization of atmospheric nitrogen, have been hypothesized to have the potential to affect insolation for periods of years, and possibly even to deplete the ozone layer¹⁶. The actual extents of these effects are all model dependent and thus poorly constrained. They cannot be specifically examined in our cores. However, we note that the revised age of 2.15 Myr places the impact close to marine oxygen-isotope stage 82. This time is significantly after the establishment of Northern Hemisphere ice sheets, but close to one of the strongest cooling events in this time period (Fig. 4). The age of the Eltanin impact is not marked by disturbances (extinctions) in the marine microfossil record, but a transient depletion of the ozone layer might have had an effect on terrestrial life.

The silicate fraction of the ejecta would be difficult, but not impossible, to detect at sites distant from the impact. Globally distributed meteoritic ejecta could amount to 0.4–25 mg cm⁻² for 1–4-km asteroids, respectively. Corresponding Ir anomalies of 0.1 ng cm⁻² for a 1-km asteroid might be undetectable, but the 6.4 ng cm⁻² of a 4-km asteroid should be measurable. If only a small fraction of the ejecta (~0.05 km³) was sediment, this would be sufficient to coat the surface of the Earth with traces of microfossils equivalent to a few diatoms per cm². Under most circumstances, we would expect such a deposit to be undetectable as these microfossils would be subject to dissolution and/or dilution by other biogenic components. Exceptions could be high-accumulation laminated sediments in the marine and lacustrine record. Other potential sites for preservation and detection of Eltanin ejecta are in the ice-free Transantarctic Mountains (TAM). These areas have been described as a cold-desert environment and thus have not been affected by strong weathering or disturbances by ice and water run-off since at least 3.8 Myr (refs 20, 21). Interestingly, rare occurrences of marine diatoms, minor quantities of radiolarians and calcareous nannofossils, and small marine sediment fragments with stratigraphic ages ranging between the Eocene and the late Pliocene have been reported from glaciogenic sediments of the TAM known as the Sirius Group deposits. But these findings were interpreted to indicate that drawdown of the East Antarctic Ice Sheet (EAIS) during the Late Pliocene led to flooding of intracratonic Antarctic basins and that the subsequent glacial advance excavated and transported the microfossils to the TAM^{22,23}. This hypothesis of unstable mid-Pliocene ice volumes has been widely debated^{24–26}, as it would require significant warming at high latitudes not observed in other studies²⁷ and it raises questions about the stability of the modern ice sheet. To resolve the Sirius enigma it was proposed that the microfossils have been imported by aeolian transport. Aeolian transport provides a viable mechanism for explaining the occurrence of freshwater^{28,29}, non-Antarctic marine²⁹ and modern Antarctic^{29,30} marine diatoms, reported on the Antarctic ice and terranes. However, the occurrence of extinct Cenozoic diatoms and other microfossils, as well as sediment fragments, that must have originated in deep-sea Southern Ocean sediments has not yet been adequately explained by the aeolian transport mechanism³¹. We suggest that an elegant solution might be that the introduction of marine microfossils and sediment was as ejecta from the Eltanin impact. This mechanism is distinct from aeolian import in that the ejecta are derived from a point source and transported above the atmosphere. A possible impact source is supported by the fact that

the Sirius microfossils fit well within the age of sediments disturbed by the impact, including upper Eocene, upper Oligocene/lower Miocene, and middle to lowermost Pliocene taxa. Many of the taxa reported in the Sirius Group till²² were found in SU III and II. The youngest diatoms, used for dating the hypothetical mid-Pliocene EAIS deglaciation (*Thalassiosira vulnifica*, *T. kolbei*), were present in surface sediments when the Eltanin impact occurred. Further research into this impact, and also into the distribution pattern and the quantity of microfossils in the TAM deposits, will test the viability of this transport mechanism. □

Received 21 January; accepted 30 September 1997.

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Acknowledgements This research was supported by Acciones Integradas grants to R.G. and J.A.F., and an NSF grant to E.T.K. and J.A.B. We thank the crew of RV *Polarstern* for their substantial support.

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The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon *Archaeoglobus fulgidus*

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***Archaeoglobus fulgidus* is the first sulphur-metabolizing organism to have its genome sequence determined. Its genome of 2,178,400 base pairs contains 2,436 open reading frames (ORFs). The information processing systems and the biosynthetic pathways for essential components (nucleotides, amino acids and cofactors) have extensive correlation with their counterparts in the archaeon *Methanococcus jannaschii*. The genomes of these two Archaea indicate dramatic differences in the way these organisms sense their environment, perform regulatory and transport functions, and gain energy. In contrast to *M. jannaschii*, *A. fulgidus* has fewer restriction-modification systems, and none of its genes appears to contain inteins. A quarter (651 ORFs) of the *A. fulgidus* genome encodes functionally uncharacterized yet conserved proteins, two-thirds of which are shared with *M. jannaschii* (428 ORFs). Another quarter of the genome encodes new proteins indicating substantial archaeal gene diversity.**

Biological sulphate reduction is part of the global sulphur cycle, ubiquitous in the earth's anaerobic environments, and is essential to the basal workings of the biosphere. Growth by sulphate reduction is restricted to relatively few groups of prokaryotes; all but one of these are Eubacteria, the exception being the archaeal sulphate reducers in the Archaeoglobales^{1,2}. These organisms are unique in that they are unrelated to other sulphate reducers, and because they grow at extremely high temperatures³. The known Archaeoglobales are strict anaerobes, most of which are hyperthermophilic marine sulphate reducers found in hydrothermal environments^{2,4} and in subsurface oil fields⁵. High-temperature sulphate reduction by *Archaeoglobus* species contributes to deep subsurface oil-well 'souring' by producing iron sulphide, which causes corrosion of iron and steel in oil- and gas-processing systems⁵.

Archaeoglobus fulgidus VC-16 (refs 2, 4) is the type strain of the Archaeoglobales. Cells are irregular spheres with a glycoprotein envelope and monopolar flagella. Growth occurs between 60 and 95 °C, with optimum growth at 83 °C and a minimum division time of 4 h. The organism grows organoheterotrophically using a variety of carbon and energy sources, but can grow lithoautotrophically on hydrogen, thiosulphate and carbon dioxide⁶. We sequenced the genome of *A. fulgidus* strain VC-16 as an example of a sulphur-metabolizing organism and to gain further insight into the Archaea^{7,8} through genomic comparison with *Methanococcus jannaschii*⁹.

General features of the genome

The genome of *A. fulgidus* consists of a single, circular chromosome of 2,178,400 base pairs (bp) with an average of 48.5% G+C content

(Fig. 1). There are three regions with low G+C content (<39%), two rich in genes encoding enzymes for lipopolysaccharide (LPS) biosynthesis, and two regions of high G+C content (>53%), containing genes for large ribosomal RNAs, proteins involved in haem biosynthesis (*hemAB*), and several transporters (Table 1). Because the origins of replication in Archaea are not characterized, we arbitrarily designated base pair one within a presumed non-coding region upstream of one of three areas containing multiple short repeat elements.

Open reading frames. Two independent coding analysis programs and BLASTX¹⁰ searches (see Methods) predicted 2,436 ORFs (Figs 1, 2, Tables 1, 2) covering 92.2% of the genome. The average size of the *A. fulgidus* ORFs is 822 bp, similar to that of *M. jannaschii* (856 bp), but smaller than that in the completely sequenced eubacterial genomes (949 bp). All ORFs were searched against a non-redundant protein database, resulting in 1,797 putative identifications that were assigned biological roles within a classification system adapted from ref. 11. Predicted start codons are 76% ATG, 22% GTG and 2% TTG. Unlike *M. jannaschii*, where 18 inteins were found in coding regions, no inteins were identified in *A. fulgidus*. Compared with *M. jannaschii*, *A. fulgidus* contains a large number of gene duplications, contributing to its larger genome size. The average protein relative molecular mass (M_r) in *A. fulgidus* is 29,753, ranging from 1,939 to 266,571, similar to that observed in other prokaryotes. The isoelectric point (pI) of predicted proteins among sequenced prokaryotes exhibits a bimodal distribution with peaks at pIs of approximately 5.5 and 10.5. The exceptions to this are *Mycoplasma genitalium* in which the distribution is skewed towards high pI

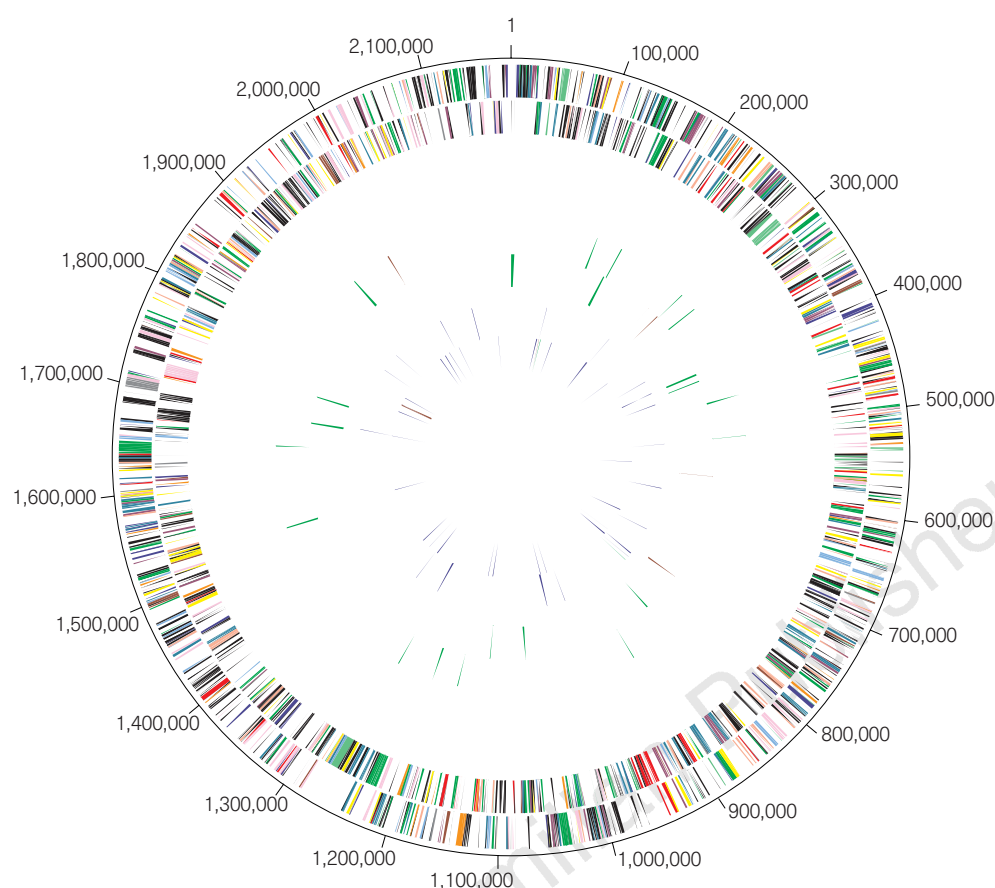


Figure 1 Circular representation of the *A. fulgidus* genome. The outer circle shows predicted protein-coding regions on the plus strand classified by function according to the colour code in Fig. 2 (except for unknowns and hypotheticals, which are in black). Second circle shows predicted protein-coding regions on the minus strand. Third and fourth circles show IS elements (red) and other repeats (green) on the plus and minus strand. Fifth and sixth circles show tRNAs (blue), rRNAs (red) and sRNAs (green) on the plus and minus strand, respectively.

Table 1 Genome features

General		
Chromosome size:	2,178,400 bp	
Protein coding regions:	92.2%	
Stable RNAs:	0.4%	
Predicted protein coding sequences:		
Identified by database match:	2,436 (1.1 per kb)	
putative function assigned:	1,797	
homologues of <i>M. jannaschii</i> ORFs:	1,096	
conserved hypothetical proteins:	916	
No database match:	651	
Members of 242 paralogous families:	639	
Members of 158 families with known functions:	719	
	475	
Stable RNAs		
	Coordinates	
16S rRNA:	1,790,478–1,788,987	
23S rRNA:	1,788,751–1,785,820	
5S rRNA:	81,144–81,021	
7S RNA:	798,067–798,376	
RNase P:	86,281–86,032	
46 species of tRNA:	no significant clusters	
tRNAs with 15–62 bp introns:	Asp ^{GUC} , Glu ^{UUC} , Leu ^{CAA} , Trp ^{CCA} , Tyr ^{GUA}	
Distinct G+C content regions		
	Coordinates	
HGC-1, >53% G+C	1,786,000–1,797,000	
HGC-2, >53% G+C	2,158,000–2,159,000	
LGC-1, <39% G+C	281,000–284,000	
LGC-2, <39% G+C	544,000–550,000	
LGC-3, <39% G+C	1,175,000–1,177,000	
Short, non-coding repeats		
	Coordinates	
SR-1A, CTTTCAATCCCATTTTGGTCTGATTCAAC	147–4,213	
SR-1B, CTTTCAATCCCATTTTGGTCTGATTCAAC	398,368–401,590	
SR-2, CTTTCAATCTCCATTTTCAGGGCCTCCCTTTCTTA	1,690,930–1,694,104	
Long, coding repeats		
	Length	Copy number
LR-01 NADH-flavin oxidoreductase	1,886 bp	2 copies
LR-02 NifS, NifU + ORF	1,549 bp	2 copies
LR-03 ISA1214 putative transposase + ISORF2	1,214 bp	6 copies
LR-04 ISA1083 putative transposase + ISORF2	1,083 bp	3 copies
LR-05 type II secretion system protein	1,014 bp	4 copies
LR-06 ISA0963 putative transposase	963 bp	7 copies
LR-07 homologue of MJ0794	836 bp	3 copies
LR-08 conserved hypothetical protein	696 bp	2 copies
LR-09 conserved hypothetical protein	628 bp	2 copies

(median, 9.8) and *A. fulgidus* where the skew is toward low pI (median, 6.3).

Multigene families. In *A. fulgidus* 719 genes (30% of the total) belong to 242 families with two or more members (Table 1). Of these families, 157 contained genes with biological roles. Most of these families contain genes assigned to the 'energy metabolism', 'transport and binding proteins', and 'fatty acid and phospholipid metabolism' categories (Table 2). The superfamily of ATP-binding subunits of ABC transporters is the largest, containing 40 members. The importance of catabolic degradation and signal recognition systems is reflected by the presence of two large superfamilies: acyl-CoA ligases and signal-transducing histidine kinases. *A. fulgidus* does not contain a homologue of the large 16-member family found in *M. jannaschii*⁹.

Repetitive elements. Three regions of the *A. fulgidus* genome contain short (<40 bp) direct repeats (Table 1). Two regions (SR-1A and SR-1B) contain 48 and 60 copies, respectively, of an identical 30-bp repeat interspersed with unique sequences averaging 40 bp. The third region (SR-2) contains 42 copies of a 37-bp repeat similar in sequence to the SR-1 repeat and interspersed with unique sequence averaging 41 bp. These repeated sequences are similar to the short repeated sequences found in *M. jannaschii*.

Nine classes of long (>500 bp) repeated sequences with ≥95% sequence identity were found (LR1-LR9; Table 1). LR-3 is a novel element with 14-bp inverted repeats and two genes, one of which has weak similarity to a transposase from *Halobacterium salinarium*. One copy of LR-3 interrupts AF2090, a homologue of a large *M. jannaschii* gene encoding a protein of unknown function. LR-4 and LR-6 encode putative transposases not identified in *M. jannaschii* that may represent IS elements. The remaining LR elements are not similar to known IS elements.

Central intermediary and energy metabolism

Sulphur oxide reduction may be the dominant respiratory process in anaerobic marine and freshwater environments, and is an important aspect of the sulphur cycle in anaerobic ecosystems¹². In this pathway, sulphate (SO_4^{2-}) is first activated to adenylylsulphate (adenosine-5'-phosphosulphate; APS), then reduced to sulphite and subsequently to sulphide^{1,13} (Fig. 3). The most important enzyme in dissimilatory sulphate reduction, adenylylsulphate reductase, reduces the activated sulphate to sulphite, releasing AMP. In *A. fulgidus*, the APS reductase has a high degree of similarity and identical physiological properties to APS reductases in sulphate-reducing delta proteobacteria¹⁴. A desulphoviridin-type sulphite reductase then adds six electrons to sulphite to produce sulphide. As in the Eubacteria, three sulphite-reductase genes, *dsrABD*, constitute an operon. The genes for adenylylsulphate reductase and sulphate adenylyltransferase reside in a separate operon. In *A. fulgidus*, sulphate can be replaced as an electron acceptor by both thiosulphate ($\text{S}_2\text{O}_3^{2-}$) and sulphite (SO_3^{2-}), but not by elemental sulphur.

A. fulgidus VC-16 has been shown to use lactate, pyruvate, methanol, ethanol, 1-propanol and formate as carbon and energy sources². Glucose has been described as a carbon source¹, but neither an uptake-transporter nor a catabolic pathway could be identified. Although it has been reported that *A. fulgidus* is incapable of growth on acetate⁶, multiple genes for acetyl-CoA synthetase (which converts acetate to acetyl-CoA) were found. The organism may degrade a variety of hydrocarbons and organic acids because of the presence of 57 β -oxidation enzymes, at least one lipase, and a minimum of five types of ferredoxin-dependent oxidoreductases (Fig. 3). The predicted β -oxidation system is similar to those in Eubacteria and mitochondria, and has not previously been described in the Archaea. *Escherichia coli* requires both the *fadD* and *fadL* gene products to import long-chain fatty acids across the cell envelope into the cytosol¹⁵. *A. fulgidus* has 14 acyl-CoA ligases related to FadD, but as expected given that it has no outer membrane, no

FadL. In *E. coli*, FadB has several metabolic functions, but in *A. fulgidus* these functions seem to be distributed among separate enzymes. For example, AF0435 encodes an orthologue of enoyl-CoA hydratase and resembles the amino-terminal domain of FadB. This gene is immediately upstream of a gene encoding an orthologue of 3-hydroxyacyl-CoA dehydrogenase that resembles the carboxy-terminal domain of FadB.

Acetyl-CoA is degraded by *A. fulgidus* through a C_1 -pathway, not by the citric acid cycle or glyoxylate bypass^{6,16,17}. This degradation is catalysed through the carbon monoxide dehydrogenase (CODH) pathway that consists of a five-subunit acetyl-CoA decarboxylase/synthase complex (ACDS) and five enzymes that are typically involved in methanogenesis¹⁸. In *A. fulgidus*, however, reverse methanogenesis occurs, resulting in CO_2 production. All of the enzymes and cofactors of methanogenesis from formylmethanofuran to N^5 -methyltetrahydromethanopterin are used, but the absence of methyl-CoM reductase eliminates the possibility of methane production by conventional pathways. Production of trace amounts of methane ($<0.1 \mu\text{mol ml}^{-1}$)¹⁹ is probably a result of the reduction of N^5 -methyltetrahydromethanopterin to methane and tetrahydromethanopterin by carbon monoxide (CO) dehydrogenase.

A. fulgidus also contains genes suggesting it has a second CO dehydrogenase system, homologous to that which enables *Rhodospirillum rubrum* to grow without light using CO as its sole energy source. Genes were detected for the nickel-containing CO dehydrogenase (CooS), an iron-sulphur redox protein, and a protein associated with the incorporation of nickel in CooS. These represent elements of a system that could catalyse the conversion of CO and H_2O to CO_2 and H_2 .

In contrast to *M. jannaschii*, *A. fulgidus* contains genes representing multiple catabolic pathways. Systems include CoA-SH-dependent ferredoxin oxidoreductases specific for pyruvate, 2-ketoisovalerate, 2-ketoglutarate and indolepyruvate, as well as a 2-oxoacid with little substrate specificity^{20,21}. Four genes with similarity to the tungsten-containing aldehyde ferredoxin oxidoreductase were also found²².

Biochemical pathways characteristic of eubacterial metabolism, including the pentose-phosphate pathway, the Entner-Doudoroff pathway, glycolysis and gluconeogenesis, are either completely absent or only partly represented (Fig. 3). *A. fulgidus* does not have typical eubacterial polysaccharide biosynthesis machinery, yet it has been shown to produce a protein and carbohydrate-containing biofilm²³. Nitrogen is obtained by importing inorganic molecules or degrading amino acids (Fig. 3); neither a glutamate dehydrogenase nor a relevant *fix* or *nif* gene is present.

The F_{420}H_2 :quinone oxidoreductase complex²⁴ is recognized as

Figure 2 Linear representation of the *A. fulgidus* genome illustrating the location of each predicted protein-coding region, RNA gene, and repeat element in the genome. Symbols for the transporters are as follows: AsO, arsenite; COH, sugar; P_i, phosphate; aa2, dipeptide; NH₄⁺, ammonium; a/o, arginine/lysine/ornithine; s/p, spermidine/putrescine; glyc, glycerol; Cl⁻, chloride; Fe²⁺, iron(II); Fe³⁺, iron(III); I, L, V, branched-chain amino acids; P, proline; pan, pantothenate; rib, ribose; lac, lactate; Mg²⁺/Co²⁺, magnesium and cobalt; gln, glutamine; NO₃⁻, nitrate; ox/for, oxalate/formate; maln, malonic acid; Hg²⁺, mercury; phs, polysaccharide; SO₄²⁻, sulphate; OCN⁻, cyanate; hex, hexuronate; phs, polysialic acid; K⁺, potassium channel; H⁺/Na⁺, sodium/proton antiporter; Na⁺/Cl⁻, sodium- and chloride-dependent transporter; P/G, osmoprotection protein; Cu²⁺, copper-transporting ATPase; +?, cation-transporting ATPase; ?, ABC-transporter without known function. Triplets associated with tRNAs represent the anticodon sequence. Numbers associated with GES represent the number of membrane-spanning domains (MSDs) according to Goldman, Engelman and Steiz scale as determined by TopPred³⁹. Genes whose identification is based on genes in *M. jannaschii* are indicated by circles. Of the 236 proteins containing at least one MSD, 124 of these had two or more MSDs.

the main generator of proton-motive force. However, our analysis indicates the presence of heterodisulphide reductase and several molybdopter-in-binding oxidoreductases, with polysulphide, nitrate, dimethyl sulphoxide, and thiosulphate as potential substrates, which might contribute to energizing the cell membrane. *A. fulgidus*

contains a large number of flavoproteins, iron-sulphur proteins and iron-binding proteins that contribute to the general intra-cellular flow of electrons (Fig. 3). Detoxification enzymes include a peroxidase/catalase, an alkyl-hydroperoxide reductase, arsenate reductase, and eight NADH oxidases, presumably catalysing the

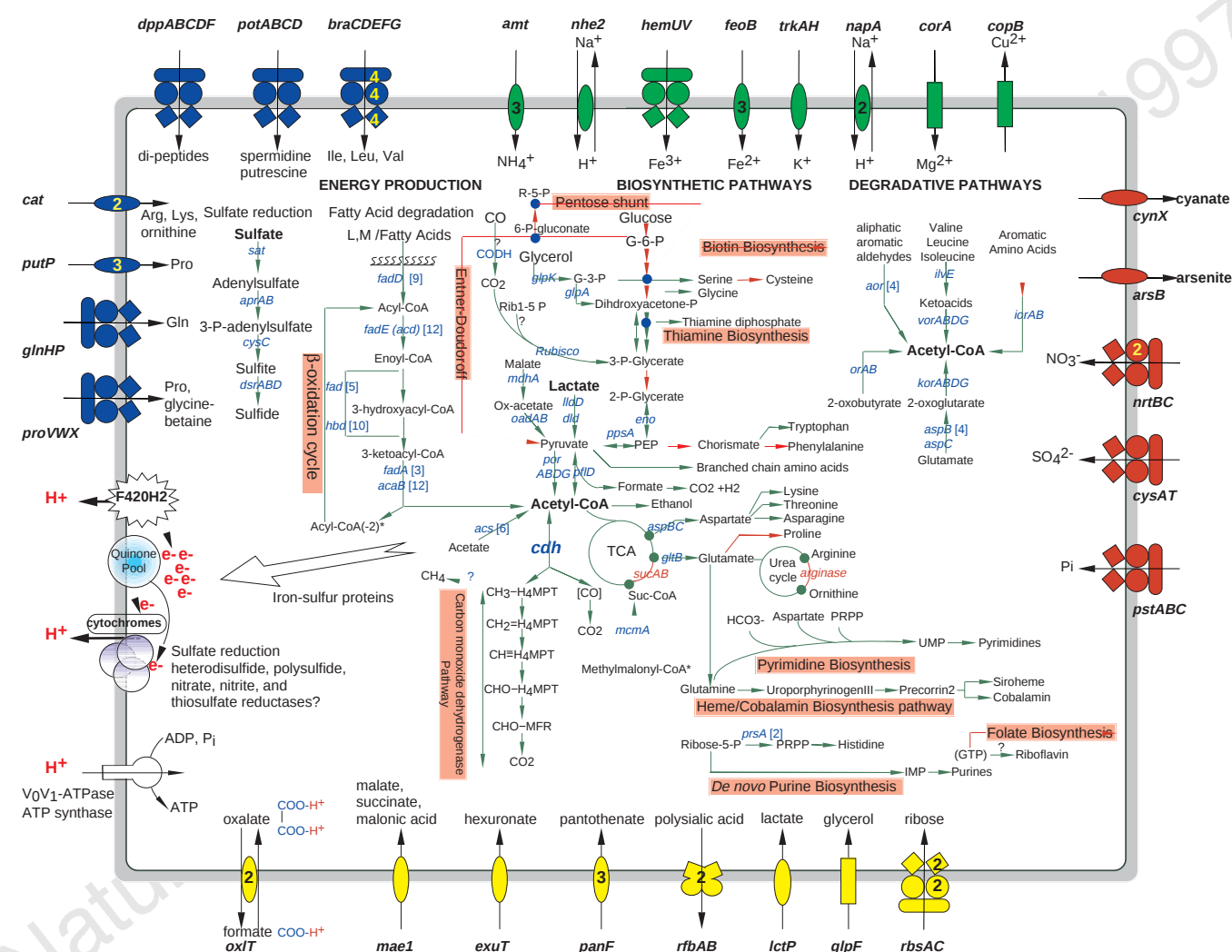


Figure 3 An integrated view of metabolism and solute transport in *A. fulgidus*. Biochemical pathways for energy production, biosynthesis of organic compounds, and degradation of amino acids, aldehydes and acids are shown with the central components of *A. fulgidus* metabolism, sulphate, lactate and acetyl-CoA highlighted. Pathways or steps for which no enzymes were identified are represented by a red arrow. A question mark is attached to pathways that could not be completely elucidated. Macromolecular biosynthesis of RNA, DNA and other lipids have been omitted. Membrane-associated reactions that establish the proton-motive force (PMF) and generate ATP (electron transport chain and V_0V_1 -ATPase) are linked to cytosolic pathways for energy production. The oxalate-formate antiporters (*oxlT*) may also contribute to the PMF by mediating electrogenic anion exchange. Each gene product with a predicted function in ion or solute transport is illustrated. Proteins are grouped by substrate specificity with transporters for cations (green), anions (red), carbohydrates/organic alcohols/acids (blue), and amino acids/peptides/amines (yellow) depicted. Ion-coupled permeases are represented by ovals (*mae1*, *exuT*, *panF*, *lctP*, *arsB*, *cynX*, *napA*/*nhe2*, *amt*, *feoB*, *trkAH*, *cat* and *putP* encode transporters for malate, hexuronate, pantothenate, lactate, arsenite, cyanate, sodium, ammonium, iron (II), potassium, arginine/lysine and proline, respectively). ATP-binding cassette (ABC) transport systems are shown as composite figures of ovals, diamonds and circles (*proVWX*, *glnHPQ*, *dppABCD*, *potABCD*, *braCDEFG*, *hemUV*, *nrtBC*, *cysAT*, *pstABC*, *rbsAC*, *rfaAB* correspond to gene products for proline, glutamine, dipeptide,

spermidine/putrescine, branch-chain amino acids, iron (III), nitrate, sulphate, phosphate, ribose and polysialic acid transport, respectively). All other porters drawn as rectangles (*glpF*, glycerol uptake facilitator; *copB*, copper transporting ATPase; *corA*, magnesium and cobalt transporter). Export and import of solutes is designated by arrows. The number of paralogous genes encoding each protein is indicated in brackets for cytoplasmic enzymes, or within the figure for transporters. Abbreviations: *acs*, acetyl-CoA synthetase; *aor*, aldehyde ferredoxin oxidoreductase; *aprAB*, adenylylsulphate reductase; *aspBC*, aspartate aminotransferase; *cdh*, acetyl-CoA decarbonylase/synthase complex; *cysC*, adenylylsulphate 3-phosphotransferase; *dld*, D-lactate dehydrogenase; *dsrABD*, sulphite reductase; *eno*, enolase; *fadA/acaB*, 3-ketoacyl-CoA thiolase; *fadD*, long-chain-fatty-acid-CoA ligase; *fad*, enoyl-CoA hydratase; *fadE (acd)*, acyl-CoA dehydrogenase; *glpA*, glycerol-3-phosphate dehydrogenase; *glpK*, glycerol kinase; *gltB*, glutamate synthase; *hbd*, 3-hydroxyacyl-CoA dehydrogenase; *ilvE*, branched-chain amino acid aminotransferase; *iorAB*, indolepyruvate ferredoxin oxidoreductase; *korABDG*, 2-ketoglutarate ferredoxin oxidoreductase; *lldD*, L-lactate dehydrogenase; *mcmA*, methylmalonyl-CoA mutase; *mdhA*, L-malate dehydrogenase; *oadAB*, oxaloacetate decarboxylase; *orAB*, 2-oxoacid ferredoxin oxidoreductase; *pflD*, pyruvate formate lyase 2; *porABDG*, pyruvate ferredoxin oxidoreductase; *ppsA*, phosphoenolpyruvate synthase; *prsA*, ribose-phosphate pyrophosphokinase; *sucAB*, 2-ketoglutarate dehydrogenase; *sat*, sulphate adenylyltransferase; TCA, tricarboxylic acid cycle; *vorABDG*, 2-ketoisovalerate ferredoxin oxidoreductase.

four-electron reduction of molecular oxygen to water, with the concurrent regeneration of NAD.

Transporters

A. fulgidus may synthesize several transporters for the import of carbon-containing compounds, probably contributing to its ability to switch from autotrophic to heterotrophic growth⁵. Both *M. jannaschii* and *A. fulgidus* have branched-chain amino-acid ABC transport systems and a transporter for the uptake of arginine and lysine. *A. fulgidus* encodes proteins for dipeptide, spermidine/putrescine, proline/glycine-betaine and glutamine uptake, as well as transporters for sugars and acids, rather like the membrane systems described in eubacterial heterotrophs. These compounds provide the necessary substrates for numerous biosynthetic and degradative pathways (Fig. 3).

Many *A. fulgidus* redox proteins are predicted to require iron. Correspondingly, iron transporters have been identified for the import of both oxidized (Fe^{3+}) and reduced (Fe^{2+}) forms of iron. There are duplications in functional and regulatory genes in both systems. The uptake of Fe^{3+} may depend on haemin or a haemin-like compound because *A. fulgidus* has orthologues to the eubacterial hem transport system proteins, HemU and HemV. *A. fulgidus* may also use the regulatory protein Fur to modulate Fe^{3+} transport; this protein is not present in *M. jannaschii*. Fe^{2+} uptake occurs through a modified Feo system containing FeoB. This is the third example of an isolated *feoB* gene: *M. jannaschii* and *Helicobacter pylori* also appear to lack *feoA*, implying that FeoA is not essential for iron transport in these organisms.

A complex suite of proteins regulates ionic homeostasis. Ten distinct transporters facilitate the flux of the physiological ions K^+ , Na^+ , NH_4^+ , Mg^{2+} , Fe^{2+} , Fe^{3+} , NO_3^- , SO_4^{2-} and inorganic phosphate (P_i). Most of these transporters have homologues in *M. jannaschii* and are therefore likely to be critical for nutrient acquisition during autotrophic growth. *A. fulgidus* has additional ion transporters for the elimination of toxic compounds including copper, cyanate and arsenite. As in *M. jannaschii*, the *A. fulgidus* genome contains two paralogous operons of cobalamin biosynthesis-cobalt transporters, *cbiMQO*.

Sensory functions and regulation of gene expression

Consistent with its extensive energy-producing metabolism and versatile system for carbon utilization, *A. fulgidus* has complex sensory and regulatory networks. These networks contain over 55 proteins with presumed regulatory functions, including members of the ArsR, AsnC and Sir2 families, as well as several iron-dependent repressor proteins. There are at least 15 signal-transducing histidine kinases, but only nine response regulators; this difference suggests there is a high degree of cross-talk between kinases and regulators. Only four response regulators appear to be in operons with histidine kinases, including those in the methyl-directed chemotaxis system (Che), which lies adjacent to the flagellar biosynthesis operon. Although rich in regulatory proteins, *A. fulgidus* apparently lacks regulators for response to amino-acid and carbon starvation as well as to DNA damage. Finally, *A. fulgidus* contains a homologue of the mammalian mitochondrial benzodiazepine receptor, which functions as a sensor in signal-transduction pathways²⁵. These receptors have been previously identified only in Proteobacteria and Cyanobacteria²⁵.

Replication, repair and cell division

A. fulgidus possesses two family B DNA polymerases, both related to the catalytic subunit of the eukaryal delta polymerase, as previously observed in the Sulfolobales²⁶. It also has a homologue of the proofreading ϵ subunit of *E. coli* Pol III, not previously observed in the Archaea. The DNA repair system is more extensive than that found in *M. jannaschii*, including a homologue of the eukaryal Rad25, a 3-methyladenine DNA glycosylase, and exodeoxynuclease

III. As well as reverse gyrase, topoisomerase I (ref. 9), and topoisomerase VI (ref. 27), the genes for the first archaeal DNA gyrase were identified.

A. fulgidus lacks a recognizable type II restriction-modification system, but contains one type I system. In contrast, two type II and three type I systems were identified in *M. jannaschii*. No homologue of the *M. jannaschii* thermonuclease was identified.

The cell-division machinery is similar to that of *M. jannaschii*, with orthologues of eubacterial *fts* and eukaryal *cdc* genes. However, several *cdc* genes found in *M. jannaschii*, including homologues of *cdc23*, *cdc27*, *cdc47* and *cdc54*, appear to be absent in *A. fulgidus*.

Transcription and translation

A. fulgidus and *M. jannaschii* have transcriptional and translational systems distinct from their eubacterial and eukaryal counterparts. In both, the RNA polymerase contains the large universal subunits and five smaller subunits found in both Archaea and eukaryotes. Transcription initiation is a simplified version of the eukaryotic mechanism^{28,29}. However, *A. fulgidus* alone has a homologue of eukaryotic TBP-interacting protein 49 not seen in *M. jannaschii*, but apparently present in *Sulfolobus solfataricus*.

Translation in *A. fulgidus* parallels *M. jannaschii* with a few exceptions. The organism has only one rRNA operon with an Ala-tRNA gene in the spacer and lacks a contiguous 5S rRNA gene. Genes for 46 tRNAs were identified, five of which contain introns in the anticodon region that are presumably removed by the intron excision enzyme EndA. The gene for selenocysteine tRNA (SelC) was not found, nor were the genes for SelA, SelB and SelD. With the exception of Asp-tRNA^{GTC} and Val-tRNA^{CAC}, tRNA genes are not linked in the *A. fulgidus* genome. The RNA component of the tRNA maturation enzyme RNase P is present. Both *A. fulgidus* and *M. jannaschii* appear to possess an enzyme that inserts the tRNA-modified nucleoside archaeosine, but only *A. fulgidus* has the related enzyme that inserts the modified base queuine.

Both *A. fulgidus* and *M. jannaschii* lack glutamine synthetase and asparagine synthetase; the relevant tRNAs are presumably aminoacylated with glutamic and aspartic acids, respectively. An enzymatic *in situ* transamidation then converts the amino acid to its amide form, as seen in other Archaea and in Gram-positive Eubacteria³⁰. Indeed, genes for the three subunits of the Glu-tRNA amidotransferase (*gatABC*) have been identified in *A. fulgidus*. The Lys aminoacyl-tRNA synthetase in both organisms is a class I-type, not a class II-type³¹. *A. fulgidus* possesses a normal tRNA synthetase for both Cys and Ser, unlike *M. jannaschii* in which the former was not identifiable and the latter was unusual⁹.

M. jannaschii has a single gene belonging to the TCP-1 chaperonin family, whereas *A. fulgidus* has two that encode subunits α and β of the thermosome. Phylogenetic analysis of the archaeal TCP-1 family indicates that these *A. fulgidus* genes arose by a recent species-specific gene duplication, as is the case for the two subunits of the *Thermoplasma acidophilum* thermosome³² and the *Sulfolobus shibatae* rosettasome³³. As in *M. jannaschii*, no *dnaK* gene was identified.

Biosynthesis of essential components

Like most autotrophic microorganisms, *A. fulgidus* is able to synthesize many essential compounds, including amino acids, cofactors, carriers, purines and pyrimidines. Many of these biosynthetic pathways show a high degree of conservation between *A. fulgidus* and *M. jannaschii*. These two Archaea are similar in their biosynthetic pathways for siroheme, cobalamin, molybdopterin, riboflavin, thiamin and nicotinate, the role category with greatest conservation between these two organisms being amino-acid biosynthesis. Of 78 *A. fulgidus* genes assigned to amino-acid biosynthetic pathways, at least 73 (94%) have homologues in *M. jannaschii*. For both archaeal species, amino-acid biosynthetic pathways resemble those of *Bacillus subtilis* more closely than

those of *E. coli*. For example, in *A. fulgidus* and *M. jannaschii*, tryptophan biosynthesis is accomplished by seven enzymes, TrpA, B, C, D, E, F, G as in *B. subtilis*, rather than by five enzymes, TrpA, B, C, D, E (including the bifunctional TrpC and TrpD) as found in *E. coli*.

No biotin biosynthetic genes were identified, yet biotin can be detected in *A. fulgidus* cell extracts³⁴, and several genes encode a biotin-binding consensus sequence. Similarly, *A. fulgidus* lacks the genes for pyridoxine biosynthesis although pyridoxine can be found in cell extracts (albeit at lower levels than seen in *E. coli* and several Archaea³⁴). No gene encoding ferrochelatase, the terminal enzyme in haem biosynthesis, has been identified, although *A. fulgidus* is known to use cytochromes³⁴. These cofactors may be obtained by mechanisms that we have not recognized. Although all of the enzymes required for pyrimidine biosynthesis appear to be present, three enzymes in the purine pathway (GAR transformylase, AICAR formyltransferase and the ATPase subunit of AIR carboxylase) have not been identified, presumably because they exist as new isoforms.

The Archaea share a unique cell membrane composed of ether lipids containing a glycerophosphate backbone with a 2,3-*sn* stereochemistry³⁵ for which there are multiple biosynthetic pathways³⁶. In the case of *Halobacterium cutirubrum*, the backbone is apparently obtained by enantiomeric inversion of *sn*-glycerol-3-phosphate; in *Sulfolobus acidocaldarius* and *Methanobacterium thermoautotrophicum*, *sn*-glycerol-1-phosphate dehydrogenase builds the backbone from dihydroxyacetonephosphate. An orthologue of *sn*-glycerol-1-phosphate dehydrogenase has been identified in *A. fulgidus*, suggesting that the latter pathway is present.

Conclusions

Although *A. fulgidus* has been studied since its discovery ten years ago¹, the completed genome sequence provides a wealth of new information about how this unusual organism exploits its environment. For example, its ability to reduce sulphur oxides has been well characterized, but genome sequence data demonstrate that *A. fulgidus* has a great diversity of electron transport systems, some of unknown specificity. Similarly, *A. fulgidus* has been characterized as a scavenger with numerous potential carbon sources, and its gene complement reveals the extent of this capability. *A. fulgidus* appears to obtain carbon from fatty acids through β -oxidation, from degradation of amino acids, aldehydes and organic acids, and perhaps from CO.

A. fulgidus has extensive gene duplication in comparison with other fully sequenced prokaryotes. For example, in the fatty acid and phospholipid metabolism category, there are 10 copies of 3-hydroxyacyl-CoA dehydrogenase, 12 copies of 3-ketoacyl-CoA thiolase, and 12 of acyl-CoA dehydrogenase. The duplicated proteins are not identical, and their presence suggests considerable metabolic differentiation, particularly with respect to the pathways for decomposing and recycling carbon by scavenging fatty acids. Other categories show similar, albeit less dramatic, gene redundancy. For example, there are six copies of acetyl-CoA synthetase and four aldehyde ferredoxin oxidoreductases for fermentation, as well as four copies of aspartate aminotransferase for amino-acid biosynthesis. These observations, together with the large number of paralogous gene families, suggest that gene duplication has been an important evolutionary mechanism for increasing physiological diversity in the Archaeoglobales.

A comparison of two archaeal genomes is inadequate to assess the diversity of the entire domain. Given this caveat, it is nevertheless possible to draw some preliminary conclusions from the comparison of *M. jannaschii* and *A. fulgidus*. A comparison of the gene content of these Archaea reveals that gene conservation varies significantly between role categories, with genes involved in transcription, translation and replication highly conserved; approximately 80% of the *A. fulgidus* genes in these categories have homologues in *M. jannaschii*. Biosynthetic pathways are also

highly conserved, with approximately 80% of the *A. fulgidus* biosynthetic genes having homologues in *M. jannaschii*. In contrast, only 35% of the *A. fulgidus* central intermediary metabolism genes have homologues, reflecting their minimal metabolic overlap.

Over half of the *A. fulgidus* ORFs (1,290) have no assigned biological role. Of these, 639 have no database match. The remaining 651, designated 'conserved hypothetical proteins', have sequence similarity to hypothetical proteins in other organisms, two-thirds with apparent homologues in *M. jannaschii*. These shared hypothetical proteins will probably add to our understanding of the genetic repertoire of the Archaea. Analysis of the *A. fulgidus* and other archaeal and eubacterial genomes will provide the information necessary to begin to define a core set of archaeal genes, as well as to better understand prokaryotic diversity. □

Methods

Whole-genome random sequencing procedure. The type strain, *A. fulgidus* VC-16, was grown from a culture derived from a single cell isolated by optical tweezers³⁷ and provided by K. O. Stetter (University of Regensburg). Cloning, sequencing and assembly were essentially as described previously for genomes sequenced by TIGR^{38–40}. One small-insert and one medium-insert plasmid library were generated by random mechanical shearing of genomic DNA. One large-insert lambda (λ) library was generated by partial *Tsp509I* digestion and ligation to λ -DASHII/*EcoRI* vector (Stratagene). In the initial random sequencing phase, 6.7-fold sequence coverage was achieved with 27,150 sequences from plasmid clones (average read length 500 bases) and 1,850 sequences from λ -clones. Both plasmid and λ -sequences were jointly assembled using TIGR assembler⁴¹, resulting in 152 contigs separated by sequence gaps and five groups of contigs separated by physical gaps. Sequences from both ends of 560 λ -clones served as a genome scaffold, verifying the orientation, order and integrity and the contigs. Sequence gaps were closed by editing the ends of sequence traces and/or primer walking on plasmid or λ -clones clones spanning the respective gap. Physical gaps were closed by combinatorial polymerase chain reaction (PCR) followed by sequencing of the PCR product. At the end of gap closure, 90 regions representing 0.33% of the genome had only single-sequence coverage. These regions were confirmed with terminator reactions to ensure a minimum of 2-fold sequence coverage for the whole genome. The final genome sequence is based on 29,642 sequences, with a 6.8-fold sequence coverage. The linkage between the terminal sequences of 2,101 clones from the small-insert plasmid library (average size 1,419 bp) and 8,726 clones from the medium-insert plasmid library (average size 2,954 bp) supported the genome scaffold formed by the λ -clones (average size 16,381 bp), with 96.9% of the genome covered by λ -clones. The reported sequence differs in 20 positions from the 14,389 bp of DNA in a total of 11 previously published *A. fulgidus* genes.

ORF prediction and gene family identification. Coding regions (ORFs) were identified using a combination strategy based on two programs. Initial sets of ORFs were derived with GeneSmith (H.O.S., unpublished), a program that evaluates ORF length, separation and overlap between ORFs, and with CRITICA (J.H.B. & G.J.O., unpublished), a coding region identification tool using comparative analysis. The two largely overlapping sets of ORFs were merged into one joint set containing all members of both initial sets. ORFs were searched against a non-redundant protein database using BLASTX¹⁰ and those shorter than 30 codons 'coding' for proteins without a database match were eliminated. Frameshifts were detected and corrected where appropriate as described previously⁴⁰. Remaining frameshifts are considered authentic and corresponding regions were annotated as 'authentic frameshift'. In total, 527 hidden Markov models, based upon conserved protein families (PFAM version 2.0), were searched with HMMER to determine ORF membership in families and superfamilies⁴². Families of paralogous genes were constructed as described previously⁴⁰. TopPred⁴³ was used to identify membrane-spanning domains in proteins.

Received 9 September; accepted 4 November 1997.

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Acknowledgements. We thank M. Heaney, J. Scott and R. Shirley for software and database support; V. Sapiro, B. Vincent, J. Meehan and D. Maas for computer system support; B. Cameron and D. J. Doyle for editorial assistance; and K. O. Stetter for providing *A. fulgidus* VC-16. This work was supported by the US Department of Energy.

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Table 2 . List of *A. fulgidus* genes with putative identification. Gene numbers correspond to those in Fig. 2. Percentages represent per cent identities.

AMINO ACID BIOSYNTHESIS			CELLULAR PROCESSES		
<i>General</i>			<i>General</i>		
AF0906	hydantoin utilization protein A (hyaA)	27.4%	AF0722	cobalamin biosynthesis precorrin-6Y methylase (cbiE)	32.4%
<i>Aromatic amino acid family</i>			AF0732	cobalamin biosynthesis precorrin-8W decarboxylase (cbiT)	30.8%
AF0228	3-dehydroquinate dehydratase (aroD)	36.8%	AF1336	cobalamin biosynthesis protein (cbiB)	38.4%
AF1497	5-enolpyruvylshikimate 3-phosphate synthase (aroA)	41.5%	AF0723	cobalamin biosynthesis protein (cbiD)	36.3%
AF1603	anthranilate synthase component I (trpE)	43.7%	AF0728	cobalamin biosynthesis protein (cbiM-1)	51.4%
AF1604	anthranilate synthase component II (trpD)	43.8%	AF1843	cobalamin biosynthesis protein (cbiM-2)	41.2%
AF1602	anthranilate synthase component II (trpG)	50.0%	AF0731	cobalt transport ATP-binding protein (cbiO-1)	47.2%
AF0227	chorismate mutase/prephenate dehydratase (pheA)	32.2%	AF1841	cobalt transport ATP-binding protein (cbiO-2)	41.1%
AF0270	chorismate synthase (aroC)	55.3%	AF0729	cobalt transport protein (cbiN)	56.0%
AF1601	phosphoribosyl anthranilate isomerase (trpF)	37.1%	AF0730	cobalt transport protein (cbiQ-1)	32.6%
AF2327	shikimate 5-dehydrogenase (aroE)	43.1%	AF1842	cobalt transport protein (cbiQ-2)	30.3%
AF0343	tryptophan repressor binding protein (wrbA)	46.6%	AF1338	cobryric acid synthase (cbP)	44.5%
AF1599	tryptophan synthase, subunit alpha (trpA)	39.5%	AF2229	cobrynic acid α , α -diamide synthase (cblA)	42.3%
AF1240	tryptophan synthase, subunit beta (trpB-1)	39.4%	AF1241	glutamate 1-semialdehyde aminotransferase (hemL)	54.3%
AF1600	tryptophan synthase, subunit beta (trpB-2)	64.1%	AF1975	glutamyl-tRNA reductase (hemA)	42.7%
<i>Aspartate family</i>			AF1594	heme biosynthesis protein (nirH)	25.2%
AF2112	5-methyltetrahydropteroyltrylgutamate-homocysteine methyltransferase (metE)	28.1%	AF1125	heme biosynthesis protein (nir-1)	38.7%
AF0882	asparaginase (asnA)	45.9%	AF2009	heme biosynthesis protein (nir-2)	31.8%
AF1439	asparagine synthetase (asnB)	36.9%	AF1593	heme d1 biosynthesis protein (nirD)	29.4%
AF2366	aspartate aminotransferase (aspB-1)	42.3%	AF1311	oxygen-independent coproporphyrinogen III oxidase, putative	27.1%
AF2129	aspartate aminotransferase (aspB-2)	45.4%	AF1242	porphobilinogen deaminase (hemC)	46.3%
AF1623	aspartate aminotransferase (aspB-3)	39.4%	AF1974	porphobilinogen synthase (hemB)	60.4%
AF0409	aspartate aminotransferase (aspB-4)	45.2%	AF1784	protoporphyrinogen oxidase (hemK)	33.5%
AF1417	aspartate aminotransferase (aspC)	46.2%	AF0422	uroporphyrin-III C-methyltransferase (cysG-1)	41.7%
AF0700	aspartate kinase (lysC)	49.1%	AF1243	uroporphyrin-III C-methyltransferase (cysG-2)	52.5%
AF1422	aspartate racemase	48.0%	AF0116	uroporphyrinogen III synthase (hemD)	27.4%
AF1506	aspartate-semialdehyde dehydrogenase (asd)	60.9%	<i>Menaquinone and ubiquinone</i>		
AF0800	diaminopimelate decarboxylase (lysA)	45.6%	AF2176	4-hydroxybenzoate octaprenyltransferase (ubiA)	41.6%
AF0747	diaminopimelate epimerase (dapF)	45.8%	AF0404	4-hydroxybenzoate octaprenyltransferase, putative	30.6%
AF0909	dihydrodipicolinate reductase (dapB)	48.6%	AF2413	coenzyme PQQ synthesis protein (pqoE)	30.5%
AF0910	dihydrodipicolinate synthase (dapA)	51.0%	AF1191	dihydroxynaphthoic acid synthase (menB)	54.6%
AF0935	homoserine dehydrogenase (hom)	47.9%	AF1551	octaprenyl-diphosphate synthase (ispB)	33.2%
AF0886	S-adenosylhomocysteine hydrolase (ahcY-1)	31.7%	AF0140	ubiquinone/menaquinone biosynthesis methyltransferase (ubiE)	31.0%
AF2000	S-adenosylhomocysteine hydrolase (ahcY-2)	67.3%	<i>Molybdopterin</i>		
AF0051	succinyl-diaminopimelate desuccinylase (dapE-1)	30.5%	AF2006	molybdenum cofactor biosynthesis protein (moaA)	47.8%
AF0904	succinyl-diaminopimelate desuccinylase (dapE-2)	43.8%	AF0265	molybdenum cofactor biosynthesis protein (moaB)	44.4%
AF0551	threonine synthase (thrC-1)	40.5%	AF2150	molybdenum cofactor biosynthesis protein (moaC)	62.0%
AF1316	threonine synthase (thrC-2)	61.0%	AF0931	molybdenum cofactor biosynthesis protein (moaE-1)	50.8%
<i>Glutamate family</i>			AF0930	molybdenum cofactor biosynthesis protein (moaE-2)	44.8%
AF1280	acetylglutamate kinase (argB)	56.1%	AF0161	molybdenum cofactor biosynthesis protein (moaE-3)	30.5%
AF2286	acetylglutamate kinase, putative	29.0%	AF0531	molybdenum cofactor biosynthesis protein (moaE)	44.0%
AF0080	acetylornithine aminotransferase (argD-1)	48.3%	AF1022	molybdenum-pterin-binding protein (mopB)	39.3%
AF1815	acetylornithine aminotransferase (argD-2)	36.2%	AF1624	molybdopterin converting factor, subunit 1 (moaD)	36.6%
AF0522	acetylornithine deacetylase (argE)	29.4%	AF2179	molybdopterin converting factor, subunit 2 (moaE)	33.3%
AF0883	argininosuccinate lyase (argH)	42.2%	AF2005	molybdopterin-guanine dinucleotide biosynthesis protein A (mobA)	33.2%
AF2252	argininosuccinate synthetase (argG)	62.0%	AF2253	molybdopterin-guanine dinucleotide biosynthesis protein B (mobB)	40.0%
AF1147	glutamate N-acetyltransferase (argI)	47.8%	<i>Pantothenate</i>		
AF0953	glutamate synthase (gltB)	57.9%	AF1645	pantothenate metabolism flavoprotein (dfr)	42.4%
AF0949	glutamine synthetase (glnA)	43.3%	<i>Riboflavin</i>		
AF2071	N-acetyl-gamma-glutamyl-phosphate reductase (argC)	53.3%	AF0494	GTP cyclohydrolase II (ribA-1)	44.5%
AF1255	ornithine carbamoyltransferase (argF)	51.7%	AF2107	GTP cyclohydrolase II (ribA-2)	47.1%
<i>Pyruvate family</i>			AF1416	riboflavin synthase (ribC)	53.3%
AF0957	2-isopropylmalate synthase (leuA-1)	53.5%	AF2128	riboflavin synthase, subunit beta (ribE)	75.9%
AF0219	2-isopropylmalate synthase (leuA-2)	53.9%	AF2007	riboflavin-specific deaminase (ribG)	43.7%
AF2199	3-isopropylmalate dehydratase, large subunit (leuC)	49.3%	<i>Thiamine</i>		
AF0629	3-isopropylmalate dehydratase, small subunit (leuD-1)	56.4%	AF2075	hydroxyethylthiazole kinase (thiM)	33.6%
AF1761	3-isopropylmalate dehydratase, small subunit (leuD-2)	57.1%	AF2208	hydroxymethylpyrimidine phosphate kinase (thiD)	35.5%
AF0628	3-isopropylmalate dehydrogenase (leuB)	59.2%	AF1695	thiamine biosynthesis protein (apbA)	36.9%
AF1720	acetoacetylase synthase, large subunit (ilv-1)	57.5%	AF2412	thiamine biosynthesis protein (thiC)	60.2%
AF1780	acetoacetylase synthase, large subunit (ilv-2)	32.1%	AF0553	thiamine biosynthesis protein (thiF)	38.1%
AF2015	acetoacetylase synthase, large subunit (ilv-3)	34.1%	AF0088	thiamine biosynthesis protein, putative	28.2%
AF2100	acetoacetylase synthase, large subunit (ilv-4)	38.4%	AF0702	thiamine biosynthetic enzyme (thiI)	50.0%
AF1719	acetoacetylase synthase, small subunit (ilvN)	60.4%	AF0733	thiamine monophosphate kinase (thiL)	30.4%
AF1672	acetoacetylase synthase, small subunit, putative	29.7%	AF2074	thiamine phosphate pyrophosphorylase (thiE)	45.5%
AF0933	branched-chain amino acid aminotransferase (ilvE)	59.0%	<i>Pyridine nucleotides</i>		
AF1014	dihydroxy-acid dehydratase (ilvD)	54.5%	AF1000	NH ₃ -dependent NAD ⁺ synthetase (nadE)	52.0%
AF1985	ketol-acid reductoisomerase (ilvC)	61.8%	AF1839	nicotinate-nucleotide pyrophosphorylase (nadC)	43.2%
<i>Serine family</i>			AF1837	quinolinate synthetase (nadA), authentic frameshift	53.9%
AF0813	phosphoglycerate dehydrogenase (serA)	48.8%	CELL ENVELOPE		
AF2138	phosphoserine phosphatase (serB)	50.7%	<i>Membranes, lipoproteins, and porins</i>		
AF0273	sarcosine oxidase, subunit alpha (soxA)	31.1%	AF1420	membrane protein	51.9%
AF0274	sarcosine oxidase, subunit beta (soxB)	26.5%	AF1354	membrane protein, putative	32.8%
AF0652	serine hydroxymethyltransferase (glyA)	56.1%	<i>Surface polysaccharides, lipopolysaccharides and antigens</i>		
<i>Histidine family</i>			AF0324	dTDP-glucose 4,6-dehydratase (rfbB)	50.0%
AF0690	ATP phosphoribosyltransferase (hisG)	31.9%	AF0043	first mannoolyl transferase (wbaZ-1)	30.0%
AF0212	histidinol dehydrogenase (hisD)	51.9%	AF0806	first mannoolyl transferase (wbaZ-2)	29.0%
AF2002	histidinol-phosphate aminotransferase (hisC-1)	39.8%	AF1728	galactosyltransferase	26.9%
AF2024	histidinol-phosphate aminotransferase (hisC-2)	36.8%	AF0044	GDP-D-mannose dehydratase (gmd-1), authentic frameshift	40.7%
AF0985	imidazoleglycerol-phosphate synthase	42.2%	AF1142	glucose-1-phosphate cytidyltransferase (rfbF)	38.6%
AF0819	imidazoleglycerol-phosphate synthase, cyclase subunit (hisF)	67.0%	AF0242	glucose-1-phosphate thymidyltransferase (graD-1)	27.7%
AF2285	imidazoleglycerol-phosphate synthase, subunit H (hisH)	44.4%	AF0325	glucose-1-phosphate thymidyltransferase (graD-2)	45.2%
AF0509	imidazoleglycerol-phosphate synthase, subunit H, putative	43.2%	AF0321	glycosyl transferase	30.7%
AF1950	phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP pyrophosphorylase (hisE)	59.6%	AF0387	glycosyltransferase, putative	33.8%
AF0713	phosphoribosyl-ATP pyrophosphorylase (hisE)	59.6%	AF0467	immunogenic protein (bcsp31-1)	34.7%
AF0986	phosphoribosyl-uridine 5-aminoimidazole carboxamide ribotide isomerase (hisA-1)	37.5%	AF0635	immunogenic protein (bcsp31-2)	44.3%
	phosphoribosyl-uridine 5-aminoimidazole carboxamide ribotide isomerase (hisA-2)	42.2%	AF0988	immunogenic protein (bcsp31-3)	28.3%
			AF0602	LPS biosynthesis protein, putative	29.6%
			AF0617	LPS biosynthesis protein, putative	29.0%
			AF0607	LPS glycosyltransferase, putative	29.7%
			AF0326	mannose-1-phosphate guanylyltransferase (rfbM), authentic frameshift	42.4%
			AF1097	mannose-6-phosphate isomerase/mannose-1-phosphate guanylyl transferase (manC)	43.1%
			AF0035	mannosephosphate isomerase, putative	31.3%
			AF0045	mannosyl transferase A (mtfA)	38.7%
			AF0311	O-antigen biosynthesis protein (rfbC), authentic frameshift	30.6%
			AF0458	phosphomannomutase (pmn)	39.5%
			AF0595	polysaccharide biosynthesis protein, putative	24.1%
			AF0322	ramnosyl transferase (rfbQ)	27.5%
			AF0323	spore coat polysaccharide biosynthesis protein (spk-2), authentic frameshift	36.3%
			AF0620	succinoglycan biosynthesis protein (exoM)	24.8%
			AF0361	UDP-glucose 4-epimerase (galE-1)	38.6%
			AF2016	UDP-glucose 4-epimerase (galE-2)	30.0%
			AF0302	UDP-glucose dehydrogenase (ugd-1)	43.8%
			AF0696	UDP-glucose dehydrogenase (ugd-2)	44.1%
			<i>Surface structures</i>		
			AF1054	flagellin (flaB-1)	30.0%
			AF1055	flagellin (flaB-2)	31.1%
			AF0275	surface layer protein B (slgB-1)	30.8%
			AF1413	surface layer protein B (slgB-2)	29.9%
			<i>Chaperones</i>		
			AF1296	small heat shock protein (hsp20-1)	52.3%
			AF1971	small heat shock protein (hsp20-2)	38.1%
			AF2238	thermosome, subunit alpha (thsA)	70.6%
			AF1451	thermosome, subunit beta (thsB)	68.2%
			<i>Chromosome-associated protein</i>		
			AF0337	archaeal histone A1 (hpyA1-1)	64.6%
			AF1493	archaeal histone A1 (hpyA1-2)	69.7%
			<i>Detoxification</i>		
			AF1217	2-nitropropane dioxygenase (ncd2)	39.7%
			AF0270	alkyl hydroperoxide reductase	73.5%
			AF1361	arsenate reductase (arsC)	40.5%
			AF0550	N-ethylmaleimide chlorohydrolase (trzA-1)	45.9%
			AF0997	N-ethylmaleimide chlorohydrolase (trzA-2)	44.5%
			AF0254	NADH oxidase (noxA-1)	35.1%
			AF0395	NADH oxidase (noxA-2)	35.5%
			AF0400	NADH oxidase (noxA-3)	40.8%
			AF0951	NADH oxidase (noxA-4)	36.7%
			AF1858	NADH oxidase (noxA-5)	34.0%
			AF0485	NADH oxidase (noxB-1)	43.3%
			AF1282	NADH oxidase (noxB-2)	42.9%
			AF0226	NADH oxidase (noxC)	38.4%
			AF0515	NADH oxidase, putative	25.5%
			AF2233	peroxidase / catalase (perA)	62.9%
			<i>Protein and peptide secretion</i>		
			AF1902	protein translocase, subunit SEC61 alpha (secY)	50.0%
			AF0536	protein translocase, subunit SEC61 gamma (secE)	25.0%
			AF2062	signal recognition particle receptor (dps)	54.8%
			AF1258	signal recognition particle, subunit SRP19 (srp19)	36.6%
			AF0622	signal recognition particle, subunit SRP54 (srp54)	51.2%
			AF1791	signal sequence peptidase (sec11)	36.3%
			AF1657	signal sequence peptidase (spc21)	47.0%
			AF1655	signal sequence peptidase, putative	34.5%
			AF0338	type II secretion system protein (gspE-1)	38.5%
			AF0659	type II secretion system protein (gspE-2)	38.2%
			AF0996	type II secretion system protein (gspE-3)	41.7%
			AF1049	type II secretion system protein (gspE-4)	46.5%
			CENTRAL INTERMEDIARY METABOLISM		
			<i>Degradation of polysaccharides</i>		
			AF1207	2-deoxy-D-glucuronate 3-dehydrogenase (kduD)	45.3%
			AF1795	endoglucanase (celM)	55.4%
			<i>Phosphorus compounds</i>		
			AF0756	exopolysphatase (ppx1)	55.1%
			<i>Polyamine biosynthesis</i>		
			AF0646	agmatinase (speB)	33.3%
			AF2334	spermidine synthase (speE)	37.1%
			<i>Polysaccharides - (cytoplasmic)</i>		
			AF0599	dolichol phosphate mannose synthase, putative	32.1%
			<i>Sulfur metabolism</i>		
			AF0288	adenylylsulfate 3-phosphotransferase (cysC)	52.0%
			AF1670	adenylylsulfate reductase, subunit A (aprA)	96.0%
			AF1669	adenylylsulfate reductase, subunit B (aprB)	97.3%
			AF1667	sulfate adenylyltransferase (sat)	28.4%
			AF2228	sulfite reductase, desulfoavidin-type subunit gamma (dsrC)	41.3%
			AF0423	sulfite reductase, subunit alpha (dsrA)	100.0%
			AF0424	sulfite reductase, subunit beta (dsrB)	100.0%
			AF0425	sulfite reductase, subunit gamma (dsrD)	97.4%
			<i>Other</i>		
			AF1706	2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase (pcbD)	29.4%
			AF0675	2-hydroxy-6-oxohepta-2,4-dienoate hydrolase (todF)	26.3%
			AF0091	2-hydroxyhepta-2,4-diene-1,7-diolate isomerase (hpcE-1)	44.5%
			AF2225	2-hydroxyhepta-2,4-diene-1,7-diolate isomerase (hpcE-2)	66.0%
			AF0333	4-hydroxyphenylacetate 3-hydroxylase (hpaA-1)	22.4%
			AF0885	4-hydroxyphenylacetate 3-hydroxylase (hpaA-2)	26.0%
			AF1027	4-hydroxyphenylacetate 3-hydroxylase (hpaA-3)	21.0%

AF1957	2-hydroxyglutaryl-CoA dehydrogenase, subunit beta (hgdB)	24.4%
AF0130	acetylpolymine aminohydrolase (aphA)	38.7%
AF2290	acetylpolymine aminohydrolase, putative	33.3%
AF0991	glutaryl-CoA dehydrogenase (gcdH)	48.7%
AF1323	group II decarboxylase	28.0%
AF2004	group II decarboxylase	46.1%
AF2295	group II decarboxylase	30.5%
AF1665	ornithine cycloaminase (arcB)	35.3%
Anaerobic		
AF1145	4-hydroxybutyrate CoA transferase (cat2-1)	46.5%
AF1854	4-hydroxybutyrate CoA transferase (cat2-2)	47.5%
AF0886	glycerol kinase (gpkK)	33.8%
AF1328	glycerol-3-phosphate dehydrogenase (gfpA)	27.8%
AF0871	glycerol-3-phosphate dehydrogenase (NAD(P) ⁺) (gpsA)	36.3%
AF0020	L-carnitine dehydratase (caib-1)	33.3%
AF0990	L-carnitine dehydratase (caib-2)	31.2%
ATP-proton motive force interconversion		
AF1158	ATP synthase, subunit E, putative	47.1%
AF1165	H ⁺ -transporting ATP synthase, subunit A (atpA)	67.0%
AF1167	H ⁺ -transporting ATP synthase, subunit B (atpB)	72.6%
AF1164	H ⁺ -transporting ATP synthase, subunit C (atpC)	37.5%
AF1168	H ⁺ -transporting ATP synthase, subunit D (atpD)	47.1%
AF1163	H ⁺ -transporting ATP synthase, subunit E (atpE)	36.3%
AF1165	H ⁺ -transporting ATP synthase, subunit F (atpF)	45.0%
AF1159	H ⁺ -transporting ATP synthase, subunit I (atpI)	30.1%
AF1160	H ⁺ -transporting ATP synthase, subunit K (atpK-1)	46.3%
AF1162	H ⁺ -transporting ATP synthase, subunit K (atpK-2)	46.3%
Electron transport		
AF2036	cytochrome C oxidase folding protein (coxD)	33.3%
AF0144	cytochrome C oxidase, subunit II (cbaB)	34.2%
AF0142	cytochrome C oxidase, subunit II, putative	38.0%
AF0190	cytochrome C oxidase, subunit II, putative	31.7%
AF1057	cytochrome C-type biogenesis protein (cobA)	30.7%
AF2192	cytochrome C-type biogenesis protein (nirE)	36.1%
AF2286	cytochrome c-ase, subunit alpha (cydA-1)	22.9%
AF2297	cytochrome oxidase, subunit I (cydA-2)	31.5%
AF2046	cytochrome oxidase, subunit I, putative	25.1%
AF0528	cytochrome-c3 hydrogenase, subunit gamma	39.3%
AF0833	desulfoferredoxin (dfr)	63.0%
AF0344	desulfoferredoxin, putative	47.3%
AF0287	electron transfer flavoprotein, subunit alpha (etfA)	39.7%
AF0286	electron transfer flavoprotein, subunit beta (etfB)	38.8%
AF1380	F420-nonreducing hydrogenase (vhtA)	34.8%
AF1371	F420-nonreducing hydrogenase (vhtD-1)	30.9%
AF1378	F420-nonreducing hydrogenase (vhtD-2)	33.1%
AF1381	F420-nonreducing hydrogenase (vhtD-3)	46.1%
AF1824	F420H2:quinone oxidoreductase, 11.2 kDa subunit, putative	24.1%
AF1823	F420H2:quinone oxidoreductase, 16.5 kDa subunit, putative	25.7%
AF1832	F420H2:quinone oxidoreductase, 32 kDa subunit (nuoI)	95.5%
AF1833	F420H2:quinone oxidoreductase, 39 kDa subunit, putative	33.6%
AF1829	F420H2:quinone oxidoreductase, 39.7 kDa subunit, putative	43.8%
AF1831	F420H2:quinone oxidoreductase, 41.2 kDa subunit, putative	34.8%
AF1827	F420H2:quinone oxidoreductase, 43.2 kDa subunit, putative	26.9%
AF1830	F420H2:quinone oxidoreductase, 45 kDa subunit (nuoD)	80.0%
AF1825	F420H2:quinone oxidoreductase, 53.9 kDa subunit (nuoM)	32.1%
AF1826	F420H2:quinone oxidoreductase, 72.4 kDa subunit (nuoL)	33.2%
AF0156	ferredoxin (fdx-1)	45.3%
AF0166	ferredoxin (fdx-2)	49.2%
AF0355	ferredoxin (fdx-3)	53.2%
AF0427	ferredoxin (fdx-4)	56.1%
AF0923	ferredoxin (fdx-5)	56.9%
AF1010	ferredoxin (fdx-6)	44.4%
AF1239	ferredoxin (fdx-7)	29.0%
AF2142	ferredoxin (fdx-8)	38.0%
AF0164	ferredoxin-nitrite reductase (nirA)	29.7%
AF2332	flavodoxin, putative	30.3%
AF0167	flavoprotein (fprA-1)	33.2%
AF1520	flavoprotein (fprA-2)	47.2%
AF0551	flavoprotein reductase	26.2%
AF1463	fumarate reductase, flavoprotein subunit (fdrA)	27.0%
AF1536	glutaredoxin (grx-1)	34.3%
AF2145	glutaredoxin (grx-2)	38.8%
AF0663	glutaredoxin reductase, subunit A (hdrA-1)	42.2%
AF1377	heterodisulfide reductase, subunit A (hdrA-2)	46.8%
AF0662	heterodisulfide reductase, subunit A/	
	methylyliologen reducing hydrogenase, subunit delta	34.2%
AF1238	heterodisulfide reductase, subunit A/methylyliologen reducing hydrogenase, subunit delta	53.7%
AF1375	heterodisulfide reductase, subunit B (hdrB)	36.0%
AF0271	heterodisulfide reductase, subunit B, putative	35.3%
AF1376	heterodisulfide reductase, subunit C (hdrC)	33.3%
AF0502	heterodisulfide reductase, subunit D, putative	33.8%
AF0809	heterodisulfide reductase, subunit D, putative	100.0%
AF0661	heterodisulfide reductase, subunit E, putative	23.8%
AF0755	heterodisulfide reductase, subunits E and D, putative	31.8%
AF0506	iron-sulfur binding reductase	38.5%
AF1773	iron-sulfur binding reductase	33.3%
AF1998	iron-sulfur binding reductase	29.6%
AF0627	iron-sulfur cluster binding protein	45.5%
AF0688	iron-sulfur cluster binding protein	44.8%
AF1153	iron-sulfur cluster binding protein	27.9%
AF1185	iron-sulfur cluster binding protein	36.7%
AF1263	iron-sulfur cluster binding protein	42.7%
AF2380	iron-sulfur cluster binding protein	36.3%
AF2381	iron-sulfur cluster binding protein	34.4%
AF2409	iron-sulfur cluster binding protein	28.2%
AF0076	iron-sulfur cluster binding protein	32.7%
AF1461	iron-sulfur cluster binding protein, putative	51.0%
AF1436	iron-sulfur flavoprotein (isf-1)	35.7%
AF1519	iron-sulfur flavoprotein (isf-2)	56.6%
AF1896	iron-sulfur flavoprotein (isf-3)	37.1%
AF1372	methylyliologen-reducing hydrogenase, subunit alpha (vhuA)	39.4%
AF1374	methylyliologen-reducing hydrogenase, subunit delta (vhuD)	41.7%
AF1373	methylyliologen-reducing hydrogenase, subunit gamma (vhuG)	38.6%
AF0157	molybdopterine oxidoreductase, iron-sulfur binding subunit	38.6%
AF0174	molybdopterine oxidoreductase, membrane subunit	26.0%
AF0175	molybdopterine oxidoreductase, iron-sulfur binding subunit	42.0%
AF0176	molybdopterine oxidoreductase, molybdopterine binding subunit	32.6%

AF0499	molybdopterine oxidoreductase, iron-sulfur binding subunit	41.5%
AF0500	molybdopterine oxidoreductase, membrane subunit	27.9%
AF1202	molybdopterine oxidoreductase, iron-sulfur binding subunit	35.5%
AF1203	molybdopterine oxidoreductase, molybdopterine binding subunit	30.1%
AF2384	molybdopterine oxidoreductase, molybdopterine binding subunit	34.6%
AF2385	molybdopterine oxidoreductase, iron-sulfur binding subunit	46.9%
AF2386	molybdopterine oxidoreductase, membrane subunit	30.3%
AF0159	molybdopterine oxidoreductase, molybdopterine binding subunit, putative	30.9%
AF2267	NAD(P)H-flavin oxidoreductase	31.4%
AF0131	NAD(P)H-flavin oxidoreductase, putative	28.2%
AF2352	NADH dehydrogenase, subunit 1, putative	28.9%
AF1828	NADH dehydrogenase, subunit 3	24.3%
AF0248	NADH-dependent flavin oxidoreductase	36.7%
AF0342	nigerythrin, putative	33.3%
AF0546	nitrate reductase, gamma subunit (narI)	30.1%
AF0501	nitrate reductase, gamma subunit, putative	29.3%
AF1126	P450 cytochrome, putative	30.5%
AF0463	polyferredoxin (mvhB), authentic frameshift	32.2%
AF1379	quinone-reactive Ni/Fe-hydrogenase B-type cytochrome subunit (hydC)	29.0%
AF0173	reductase, assembly protein	30.0%
AF0547	reductase, iron-sulfur binding subunit	28.3%
AF0867	reductase, putative	33.3%
AF0880	rubredoxin (rd-1)	69.2%
AF1349	rubredoxin (rd-2)	67.9%
AF0832	rubrythrin (rr1)	45.7%
AF0831	rubrythrin (rr2)	63.7%
AF1640	rubrythrin (rr3)	37.8%
AF2312	rubrythrin (rr4)	41.4%
AF0711	thioredoxin (trx-1)	28.4%
AF0769	thioredoxin (trx-2)	38.5%
AF1284	thioredoxin (trx-3)	52.9%
AF2144	thioredoxin (trx-4)	48.9%
AF1339	ubiquinol-cytochrome C reductase complex, subunit VI requiring protein	60.9%
Fermentation		
AF1779	2-hydroxyacid dehydrogenase, putative	37.6%
AF0469	2-ketoglutarate ferredoxin oxidoreductase, subunit alpha (korA)	52.3%
AF0468	2-ketoglutarate ferredoxin oxidoreductase, subunit beta (korB)	51.2%
AF0470	2-ketoglutarate ferredoxin oxidoreductase, subunit delta (korD)	47.2%
AF0471	2-ketoglutarate ferredoxin oxidoreductase, subunit gamma (korG)	40.0%
AF2053	2-ketoglutarate ferredoxin oxidoreductase, subunit alpha (vorA)	41.2%
AF2052	2-ketoglutarate ferredoxin oxidoreductase, subunit beta (vorB)	42.7%
AF2054	2-ketoglutarate ferredoxin oxidoreductase, subunit delta (vorD)	51.5%
AF2055	2-ketoglutarate ferredoxin oxidoreductase, subunit gamma (vorG)	45.2%
AF0749	2-oxoacid ferredoxin oxidoreductase, subunit alpha (ora)	33.7%
AF0750	2-oxoacid ferredoxin oxidoreductase, subunit beta (orb)	49.2%
AF1286	acetoacetyl-CoA synthetase, putative	35.1%
AF0197	acetyl-CoA synthetase (acs-1)	27.1%
AF0366	acetyl-CoA synthetase (acs-2)	47.3%
AF0677	acetyl-CoA synthetase (acs-3)	40.9%
AF0975	acetyl-CoA synthetase (acs-4)	42.3%
AF0976	acetyl-CoA synthetase (acs-5)	36.2%
AF1287	acetyl-CoA synthetase (acs-6)	34.3%
AF0924	alcohol dehydrogenase, iron-containing	36.2%
AF0339	alcohol dehydrogenase, iron-containing	37.4%
AF2019	alcohol dehydrogenase, iron-containing	35.7%
AF2389-1	acetyl-CoA synthetase, putative	64.8%
AF2389-N	acetyl-CoA synthetase, putative	59.3%
AF2101	alcohol dehydrogenase, zinc-dependent	34.8%
AF0023	aldehyde ferredoxin oxidoreductase (aor-1)	41.1%
AF0077	aldehyde ferredoxin oxidoreductase (aor-2)	32.6%
AF0340	aldehyde ferredoxin oxidoreductase (aor-3)	38.4%
AF2281	aldehyde ferredoxin oxidoreductase (aor-4)	53.0%
AF0006	carboxymethyltransferase protein (mtaC-1)	30.7%
AF0001	carboxymethyltransferase protein (mtaC-2)	29.5%
AF0394	D-formate dehydrogenase, cytochrome-type (dld)	31.9%
AF0560	D-formate dehydrogenase (fdhH1), authentic frameshift	32.9%
AF1199	glutamate CoA-transferase, subunit A (gctA)	31.9%
AF1198	glutamate CoA-transferase, subunit B (gctB)	37.0%
AF1489	indolepyruvate ferredoxin oxidoreductase, subunit alpha (iorA)	48.1%
AF2030	indolepyruvate ferredoxin oxidoreductase, subunit beta (iorB)	41.1%
AF0807	L-lactate dehydrogenase, cytochrome-type (lldD)	39.4%
AF0865	L-malate dehydrogenase, NAD ⁺ -dependent (mdtA)	40.1%
AF2085	oxaloacetate decarboxylase, biotin carboxyl carrier subunit, putative	38.7%
AF2084	oxaloacetate decarboxylase, sodium ion pump subunit (oadB)	59.8%
AF1252	oxaloacetate decarboxylase, subunit alpha (oadA)	63.3%
AF1701	pyruvate ferredoxin oxidoreductase, subunit alpha (porA)	50.3%
AF1702	pyruvate ferredoxin oxidoreductase, subunit beta (porB)	50.7%
AF1700	pyruvate ferredoxin oxidoreductase, subunit delta (porD)	53.1%
AF1699	pyruvate ferredoxin oxidoreductase, subunit gamma (porG)	50.8%
Gluconeogenesis		
AF0710	phosphoenolpyruvate synthase (ppsA)	61.4%
Glycolysis		
AF1146	3-phosphoglycerate kinase (pgk)	48.8%
AF1132	enolase (eno)	53.9%
AF1732	glyceraldehyde 3-phosphate dehydrogenase (gap)	56.6%
AF1304	triosephosphate isomerase (tpiA)	56.4%
Pentose phosphate pathway		
AF0943	ribose 5-phosphate isomerase (rpi)	48.9%
Sugars		
AF0356	carbohydrate kinase, pfkB family	31.3%
AF0401	carbohydrate kinase, pfkB family	34.1%
AF1324	carbohydrate kinase, FGGY family	27.1%
AF1752	carbohydrate kinase, FGGY family	29.3%
AF0861	D-arabino 3-hexulose 6-phosphate formaldehyde lyase (hps-1)	30.6%
AF1305	D-arabino 3-hexulose 6-phosphate formaldehyde lyase (hps-2)	44.2%
AF0480	fructose-1-phosphate aldolase (fucA)	31.8%

TCA cycle		
AF1963	aconitase (acn)	57.1%
AF1340	citrate synthase (citZ)	50.3%
AF1098	fumarate (fum-1)	49.1%
AF1099	fumarate (fum-2)	53.4%
AF0647	isocitrate dehydrogenase, NADP (icd)	57.2%
AF1727	malate oxidoreductase (mae)	52.3%
AF0681	succinate dehydrogenase, flavoprotein subunit A	48.2%
AF0682	succinate dehydrogenase, iron-sulfur subunit B (sdhB)	53.3%
AF0683	succinate dehydrogenase, subunit C (sdhC)	36.6%
AF0684	succinate dehydrogenase, subunit D (sdhD)	25.9%
AF1539	succinyl-CoA synthetase, alpha subunit (sucD-1)	56.9%
AF2185	succinyl-CoA synthetase, alpha subunit (sucD-2)	63.5%
AF1540	succinyl-CoA synthetase, beta subunit (sucC-1)	51.3%
AF2186	succinyl-CoA synthetase, beta subunit (sucC-2)	49.6%
FATTY ACID AND PHOSPHOLIPID METABOLISM		
General		
AF1736	3-hydroxy-3-methylglutaryl-coenzyme A reductase (mvbA)	57.1%
AF0017	3-hydroxyacyl-CoA dehydrogenase (hbd-1)	41.1%
AF0285	3-hydroxyacyl-CoA dehydrogenase (hbd-2)	55.8%
AF0434	3-hydroxyacyl-CoA dehydrogenase (hbd-3)	40.7%
AF1025	3-hydroxyacyl-CoA dehydrogenase (hbd-4)	45.6%
AF1122	3-hydroxyacyl-CoA dehydrogenase (hbd-5)	45.2%
AF1177	3-hydroxyacyl-CoA dehydrogenase (hbd-6)	35.8%
AF1190	3-hydroxyacyl-CoA dehydrogenase (hbd-7)	46.5%
AF1206	3-hydroxyacyl-CoA dehydrogenase (hbd-8)	36.3%
AF2017	3-hydroxyacyl-CoA dehydrogenase (hbd-9)	35.4%
AF2273	3-hydroxyacyl-CoA dehydrogenase (hbd-10)	39.4%
AF0018	3-ketoacyl-CoA thiolase (acaB-1)	41.0%
AF0034	3-ketoacyl-CoA thiolase (acaB-2)	38.3%
AF0133	3-ketoacyl-CoA thiolase (acaB-3)	32.3%
AF0134	3-ketoacyl-CoA thiolase (acaB-4)	32.5%
AF0201	3-ketoacyl-CoA thiolase (acaB-5)	26.9%
AF0202	3-ketoacyl-CoA thiolase (acaB-6)	33.5%
AF0283	3-ketoacyl-CoA thiolase (acaB-7)	42.0%
AF0438	3-ketoacyl-CoA thiolase (acaB-8)	42.4%
AF0967	3-ketoacyl-CoA thiolase (acaB-9)	33.7%
AF0968	3-ketoacyl-CoA thiolase (acaB-10)	28.0%
AF1291	3-ketoacyl-CoA thiolase (acaB-11)	40.1%
AF2416	3-ketoacyl-CoA thiolase (acaB-12)	49.8%
AF1028	3-ketoacyl-CoA thiolase (fadA-1)	38.8%
AF1197	3-ketoacyl-CoA thiolase (fadA-2)	47.2%
AF2243	3-ketoacyl-CoA thiolase (fadA-3)	40.3%
AF0033	acyl carrier protein synthase (acaA-1)	28.6%
AF2415	acyl carrier protein synthase (acaA-2)	58.7%
AF0199	acyl-CoA dehydrogenase (acd-1)	35.9%
AF0436	acyl-CoA dehydrogenase (acd-2)	44.1%
AF0498	acyl-CoA dehydrogenase (acd-3)	22.9%
AF0671	acyl-CoA dehydrogenase (acd-4)	37.9%
AF0845	acyl-CoA dehydrogenase (acd-5)	44.8%
AF0964	acyl-CoA dehydrogenase (acd-6)	35.8%
AF1026	acyl-CoA dehydrogenase (acd-7)	42.8%
AF1141	acyl-CoA dehydrogenase (acd-8)	43.2%
AF1293	acyl-CoA dehydrogenase (acd-9)	45.8%
AF2057	acyl-CoA dehydrogenase (acd-10)	44.6%
AF2244	acyl-CoA dehydrogenase (acd-11)	42.6%
AF2275	acyl-CoA dehydrogenase (acd-12)	38.9%
AF1175	acyl-CoA dehydrogenase, short chain-specific (acdS)	30.1%
AF0818	acylphosphatase (acyP)	36.8%
AF0868	alkylidihydroxyacetonephosphate synthase	33.6%
AF2286	bifunctional short chain isoprenyl diphosphate synthase (idsA)	42.7%
AF0220	biotin carboxylase (acc)	59.1%
AF0865	carboxylesterase (est-1)	27.1%
AF1537	carboxylesterase (est-2)	29.0%
AF2336	carboxylesterase (est-3)	30.4%
AF1716	carboxylesterase (estA)	40.4%
AF1744	CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase (pgsA-2)	26.7%
AF1143	CDP-diacylglycerol-glycerol-3-phosphate-3-phosphatidyltransferase (pgsA-1)	27.0%
AF2044	CDP-diacylglycerol-serine O-phosphatidyltransferase (pssA)	36.8%
AF0435	enoyl-CoA hydratase (fad-1)	47.8%
AF0885	enoyl-CoA hydratase (fad-2)	39.9%
AF0963	enoyl-CoA hydratase (fad-3)	48.6%
AF1641	enoyl-CoA hydratase (fad-4)	41.7%
AF2429	enoyl-CoA hydratase (fad-5)	34.7%
AF1763	lipase, putative	33.5%
AF0089	long-chain-fatty-acyl-CoA ligase (fadD-1)	31.9%
AF0200	long-chain-fatty-acyl-CoA ligase (fadD-2)	34.8%
AF0962	long-chain-fatty-acyl-CoA ligase (fadD-3)	39.9%
AF0840	long-chain-fatty-acyl-CoA ligase (fadD-4)	38.1%
AF1029	long-chain-fatty-acyl-CoA ligase (fadD-5)	37.8%
AF1510	long-chain-fatty-acyl-CoA ligase (fadD-6)	36.0%
AF1772	long-chain-fatty-acyl-CoA ligase (fadD-7)	38.7%
AF1932	long-chain-fatty-acyl-CoA ligase (fadD-8)	31.0%
AF2368	long-chain-fatty-acyl-CoA ligase (fadD-9)	38.7%
AF1753	lysophospholipase	33.5%
AF0196	medium-chain acyl-CoA ligase (alkK-1)	34.6%
AF0262	medium-chain acyl-CoA ligase (alkK-2)	38.6%
AF1572	medium-chain acyl-CoA ligase (alkK-3)	31.0%
AF1591	medium-chain acyl-CoA ligase (alkK-4)	42.7%
AF2033	medium-chain acyl-CoA ligase (alkK-5)	33.5%
AF2289	myo-inositol kinase (mvk)	40.2%
AF1794	methyl-inositol-1-phosphate synthase (ino1)	32.6%
AF0245	phosphatidylserine decarboxylase (psd2)	44.5%
AF1674	sn-glycerol-1-phosphate dehydrogenase (gldA)	42.0%
AUTOTROPHIC METABOLISM		
General		
AF1100	acetyl-CoA decarboxylase/synthase, subunit alpha (cdhA-1)	50.4%
AF2397	acetyl-CoA decarboxylase/synthase, subunit alpha (cdhA-2)	54.0%
AF0379	acetyl-CoA decarboxylase/synthase, subunit beta (cdhC)	62.7%
AF0377	acetyl-CoA decarboxylase/synthase, subunit delta (cdhD)	57.4%
AF1101	acetyl-CoA decarboxylase/synthase, subunit epsilon (cdhB-1)	40.0%
AF2398	acetyl-CoA decarboxylase/synthase, subunit epsilon (cdhB-2)	38.9%
AF0376	acetyl-CoA decarboxylase/synthase, subunit gamma (cdhE)	55.4%
AF1849	carbon monoxide dehydrogenase, catalytic subunit (cooS)	39.9%
AF0950	carbon monoxide dehydrogenase, iron sulfur subunit (cooF)	38.9%
AF1535	ferredoxin-thioredoxin reductase, catalytic subunit (ftrB)	38.6%
AF2073	formylmethanofuran:tetrahydromethanopterin formyltransferase (ftr-1)	46.0%
AF2207	formylmethanofuran:tetrahydromethanopterin	49.8%

AF1935	N5,N10-methylenetetrahydromethanopterin cyclohydrolase (mch)	97.3%	AF0004	RNase I inhibitor	54.5%	AF0633	isoleucyl-tRNA synthetase (ileS)	48.9%		
AF0714	N5,N10-methylenetetrahydromethanopterin dehydrogenase (mtd)	61.8%	AF0021	signal-transducing histidine kinase	26.1%	AF2421	leucyl-tRNA synthetase (leuS)	49.7%		
AF1066	N5,N10-methylenetetrahydromethanopterin reductase (mer-1)	59.1%	AF0208	signal-transducing histidine kinase	27.9%	AF1216	lysyl-tRNA synthetase (lysS)	43.6%		
AF1196	N5,N10-methylenetetrahydromethanopterin reductase (mer-2)	37.4%	AF0450	signal-transducing histidine kinase	32.4%	AF1453	methionyl-tRNA synthetase (metS)	45.2%		
AF0009	N5-methyltetrahydromethanopterin:coenzyme M methyltransferase (mtm)	42.1%	AF0770	signal-transducing histidine kinase	26.9%	AF1955	phenylalanyl-tRNA synthetase, subunit alpha (pheS)	44.4%		
AF1587	ribulose biphosphate carboxylase, large subunit (rbcL-1)	40.6%	AF0893	signal-transducing histidine kinase	28.7%	AF1424	phenylalanyl-tRNA synthetase, subunit beta (pheT)	42.6%		
AF1638	ribulose biphosphate carboxylase, large subunit (rbcL-2)	44.9%	AF1184	signal-transducing histidine kinase	29.8%	AF1609	prolyl-tRNA synthetase (proS)	56.8%		
AF1930	tungsten formylmethanofuran dehydrogenase, subunit A (fwdA)	48.9%	AF1452	signal-transducing histidine kinase	28.5%	AF2035	seryl-tRNA synthetase (serS)	45.4%		
AF1650	tungsten formylmethanofuran dehydrogenase, subunit B (fwdB-1)	37.0%	AF1467	signal-transducing histidine kinase	37.4%	AF0548	threonyl-tRNA synthetase (thrS)	46.3%		
AF1929	tungsten formylmethanofuran dehydrogenase, subunit B (fwdB-2)	49.4%	AF1472	signal-transducing histidine kinase	30.4%	AF1694	tyrosyl-tRNA synthetase (tyrS)	52.4%		
AF1931	tungsten formylmethanofuran dehydrogenase, subunit C (fwdC)	44.1%	AF1515	signal-transducing histidine kinase	27.7%	AF0776	tyrosyl-tRNA synthetase (tyrS)	57.6%		
AF1651	tungsten formylmethanofuran dehydrogenase, subunit D (fwdD-1)	32.6%	AF1639	signal-transducing histidine kinase	29.9%	AF2224	valyl-tRNA synthetase (valS)	54.5%		
AF1928	tungsten formylmethanofuran dehydrogenase, subunit D (fwdD-2)	52.6%	AF1721	signal-transducing histidine kinase	34.5%	Degradation of proteins, peptides, and glycopeptides				
AF0177	tungsten formylmethanofuran dehydrogenase, subunit E (fwdE)	29.7%	AF2109	signal-transducing histidine kinase	31.6%	AF1976	26S protease regulatory subunit 4	66.0%		
AF1644	tungsten formylmethanofuran dehydrogenase, subunit F (fwdF)	38.2%	AF0881	signal-transducing histidine kinase, authentic frameshift	26.5%	AF1653	alkaline serine protease (aprM)	44.5%		
AF1649	tungsten formylmethanofuran dehydrogenase, subunit G (fwdG)	45.6%	AF0277	signal-transducing histidine kinase, putative	29.8%	AF0578	aminopeptidase, putative	27.9%		
PURINES, PYRIMIDINES, NUCLEOSIDES, AND NUCLEOTIDES					AF0410	signal-transducing histidine kinase, putative	27.1%	AF0364	ATP-dependent protease La (lon)	36.6%
2'-Deoxyribonucleotide metabolism					AF0448	signal-transducing histidine kinase, putative	26.1%	AF1946	cysteine proteinase, putative	36.2%
AF1108	deoxycytidine triphosphate deaminase, putative	38.1%	AF1482	signal-transducing histidine kinase, putative	25.2%	AF1281	intracellular protease (ppl)	56.0%		
AF1664	ribonucleotide reductase (nrd)	59.7%	AF0202	signal-transducing histidine kinase, putative	22.5%	AF1112	O-sialoglycoprotein endopeptidase (gcp)	57.6%		
AF1554	thioredoxin reductase (trxB)	45.2%	AF2420	signal-transducing histidine kinase, putative	28.4%	AF0665	O-sialoglycoprotein endopeptidase, putative	56.3%		
AF0247	thymidylate synthase, putative	33.1%	AF0442	succinoyl-glycan biosynthesis regulator (exsB)	37.2%	AF2086	protease inhibitor, putative	37.0%		
Nucleotide and nucleoside interconversions					AF1516	sugar fermentation stimulation protein (sfsA)	31.0%	AF0490	proteasome, subunit alpha (psmA)	60.8%
AF0876	5'-nucleotidase (ntf)	30.9%	AF1270	transcriptional regulatory protein, ArsR family	35.4%	AF0481	proteasome, subunit beta (psmB)	58.3%		
AF0676	adenylate kinase (ack)	56.1%	AF1544	transcriptional regulatory protein, ArsR family	32.3%	AF2034	X-pro aminopeptidase (pepQ)	34.6%		
AF1900	cytidylate kinase (cmk)	48.6%	AF1853	transcriptional regulatory protein, ArsR family	34.9%	Protein modification				
AF0767	nucleoside diphosphate kinase (ndk)	56.4%	AF2136	transcriptional regulatory protein, ArsR family	39.8%	AF0656	antibiotic maturation protein (pmbA)	32.7%		
AF0061	thymidylate kinase (tmk)	34.9%	AF0439	transcriptional regulatory protein, AsnC family	29.8%	AF0378	CODH nickel-insertion accessory protein (cocC-1)	35.7%		
AF1308	thymidylate kinase, putative	26.3%	AF0474	transcriptional regulatory protein, AsnC family	51.0%	AF1685	CODH nickel-insertion accessory protein (cocC-2)	47.4%		
AF2042	uridylate kinase (pyrH)	53.6%	AF0584	transcriptional regulatory protein, AsnC family	35.3%	AF1615	cofactor modifying protein (cmo)	27.2%		
Purine ribonucleotide biosynthesis					AF1121	transcriptional regulatory protein, AsnC family	35.8%	AF2195	deoxyhypusine synthase (dys1-1)	32.6%
AF2242	adenylosuccinate lyase (purB)	52.3%	AF1404	transcriptional regulatory protein, AsnC family	32.6%	AF2200	deoxyhypusine synthase (dys1-2)	34.9%		
AF0841	adenylosuccinate synthetase (purA)	70.8%	AF1448	transcriptional regulatory protein, AsnC family	45.1%	AF0381	diphthine synthase (dphS)	40.8%		
AF0873	amidophosphoribosyltransferase (purF)	55.8%	AF1428	transcriptional regulatory protein, AsnC family	30.6%	AF2324	fmu and fmv protein	40.0%		
AF0253	GMP synthase (guaA-1)	59.8%	AF1743	transcriptional regulatory protein, AsnC family	46.4%	AF1367	hydrogenase expression/formation protein (hvpA)	40.4%		
AF1320	GMP synthase (guaA-2)	49.4%	AF1283	transcriptional regulatory protein, AsnC family	46.4%	AF1388	hydrogenase expression/formation protein (hvpB)	54.4%		
AF1911	inosine monophosphate cyclohydrolase	38.3%	AF1743	transcriptional regulatory protein, AsnC family	34.9%	AF1389	hydrogenase expression/formation protein (hvpC)	40.5%		
AF0847	inosine monophosphate dehydrogenase (guaB-1)	41.6%	AF2127	transcriptional regulatory protein, LysR family	30.8%	AF1370	hydrogenase expression/formation protein (hvpD)	46.0%		
AF2118	inosine monophosphate dehydrogenase (guaB-2)	31.9%	AF0114	transcriptional regulatory protein, putative	35.6%	AF1385	hydrogenase expression/formation protein (hvpE)	51.5%		
AF1259	inosine monophosphate dehydrogenase, putative	51.6%	AF1988	transcriptional regulatory protein, Rok family	32.9%	AF1386	hydrogenase expression/formation regulatory protein (hvpF)	45.1%		
AF1157	phosphoribosylamine-glycine lyase (purD)	40.9%	AF1112	transcriptional regulatory protein, Sir2 family	38.9%	AF0036	L-isocaparyl protein carboxyl methyltransferase (pcm-1)	60.7%		
AF1271	phosphoribosylaminoimidazole carboxylase (purE)	42.8%	AF1676	transcriptional regulatory protein, Sir2 family	40.8%	AF2322	L-isocaparyl protein carboxyl methyltransferase (pcm-2)	59.3%		
AF1272	phosphoribosylaminoimidazolesuccinocarboxamide synthase (purC)	34.7%	AF1817	transcriptional regulatory protein, TetR family	34.5%	AF1840	methionyl aminopeptidase (map)	48.6%		
AF1693	phosphoribosylformylglycinamide cyclo-lyase (purM)	53.8%	AF0363	transcriptional repressor (cinR)	27.5%	AF1989	peptidyl-prolyl cis-trans isomerase (slyD)	34.4%		
AF1280	phosphoribosylformylglycinamide synthase I (purO)	40.9%	REPLICATION			AF0853	proliferating-cell nuclear antigen P120, putative	35.7%		
AF1940	phosphoribosylformylglycinamide synthase II (purI)	41.5%	AF2117	3-methyladenine DNA glycosylase (alkA)	30.0%	AF2039	proliferating-cell nuclear antigen P120, putative	44.2%		
AF0589	ribose-phosphate pyrophosphokinase (prsA-1)	35.0%	AF2060	activator 1, replication factor C, 35 KDa subunit	66.3%	AF1449	pyruvate formate-lyase 2 (pflD)	37.8%		
AF1419	ribose-phosphate pyrophosphokinase (prsA-2)	41.1%	AF1195	activator 1, replication factor C, 53 KDa subunit	43.7%	AF1450	pyruvate formate-lyase 2 activating enzyme (pflC)	38.8%		
Pyrimidine ribonucleotide biosynthesis					AF0465	DNA gyrase, subunit A (gyrA)	48.4%	AF0117	pyruvate formate-lyase activating enzyme (act-1)	25.5%
AF0106	aspartate carbamoyltransferase, catalytic subunit (pyrB)	60.7%	AF0530	DNA gyrase, subunit B (gyrB)	58.4%	AF0918	pyruvate formate-lyase activating enzyme (act-2)	42.3%		
AF0107	aspartate carbamoyltransferase, regulatory subunit (pyrI)	48.2%	AF1388	DNA helicase, putative	46.8%	AF2200	pyruvate formate-lyase activating enzyme (act-3)	45.9%		
AF1274	carbamoyl-phosphate synthase, large subunit (carB)	65.1%	AF1676	DNA helicase, putative	44.4%	AF2278	pyruvate formate-lyase activating enzyme (act-4)	50.2%		
AF1273	carbamoyl-phosphate synthase, small subunit (carA)	55.2%	AF0623	DNA ligase (lig)	32.7%	AF0380	transmembrane oligosaccharyl transferase, putative	27.8%		
AF0252	CTP synthase (pyrG)	58.3%	AF1725	DNA ligase, putative	32.7%	AF0329	transmembrane oligosaccharyl transferase, putative	29.3%		
AF2250	dihydroorotase (pyrC)	37.2%	AF0497	DNA polymerase B1 (polB)	45.1%	Ribosomal proteins: synthesis and modification				
AF0745	dihydroorotase dehydrogenase (pyrD)	44.8%	AF0683	DNA polymerase B2 (boxA), authentic frameshift	32.3%	AF1490	LSU ribosomal protein L1P (rpL1P)	48.6%		
AF1741	orotate phosphoribosyl transferase (pyrE)	49.0%	AF0972	DNA polymerase III, subunit epsilon (dnaG)	31.9%	AF1922	LSU ribosomal protein L2P (rpL2P)	60.4%		
AF0386	orotate phosphoribosyl transferase, putative	49.0%	AF2277	DNA polymerase, bacteriophage-type	36.9%	AF1925	LSU ribosomal protein L3P (rpL3P)	56.5%		
Salvage of nucleosides and nucleotides					AF0742	DNA primase, putative	26.8%	AF1924	LSU ribosomal protein L4P (rpL4P)	56.4%
AF0240	adenine deaminase (adeC)	39.5%	AF0264	DNA repair protein RAD2 (rad2)	44.4%	AF1912	LSU ribosomal protein L5P (rpL5P)	51.7%		
AF1764	dCMP deaminase, putative	39.0%	AF0358	DNA repair protein RAD25	32.5%	AF1909	LSU ribosomal protein L6P (rpL6P)	53.7%		
AF1788	methylothioadenosine phosphorylase (mtaP)	40.0%	AF1031	DNA repair protein RAD32 (rad32)	37.6%	AF0764	LSU ribosomal protein L7AE (rpL7AE)	60.7%		
AF1341	thymidine phosphorylase (deoA-1)	46.7%	AF0363	DNA repair protein REC	40.0%	AF1491	LSU ribosomal protein L10E (rpL10E)	45.6%		
AF1342	thymidine phosphorylase (deoA-2)	40.7%	AF2418	DNA repair protein, putative	28.9%	AF0538	LSU ribosomal protein L11P (rpL11P)	67.8%		
AF0239	xanthine-guanine phosphoribosyltransferase (gptA-1)	25.7%	AF1806	DNA topoisomerase I (topA)	36.2%	AF1492	LSU ribosomal protein L12A (rpL12A)	76.0%		
AF1789	xanthine-guanine phosphoribosyltransferase (gptA-2)	28.2%	AF0940	DNA topoisomerase VI, subunit A (top6A)	39.8%	AF1258	LSU ribosomal protein L13P (rpL13P)	47.4%		
REGULATORY FUNCTIONS					AF0652	DNA topoisomerase VI, subunit B (top6B)	43.9%	AF1915	LSU ribosomal protein L14P (rpL14P)	66.7%
AF1959	(R)-hydroxylglutaryl-CoA dehydratase activator (hgdC)	51.2%	AF1692	endonuclease III (ntf)	44.3%	AF2319	LSU ribosomal protein L15E (rpL15E)	70.3%		
AF0168	arsenical resistance operon repressor, putative	36.7%	AF2314	methylated-DNA-protein-cysteine methyltransferase (ogt)	55.3%	AF1903	LSU ribosomal protein L15P (rpL15P)	53.8%		
AF2204	arylsulfatase regulatory protein, putative	29.9%	AF1409	modification methylase, type III R/M system	31.4%	AF1127	LSU ribosomal protein L18E (rpL18E)	53.8%		
AF0074	biotin operon repressor/biotin-[acetyl CoA carboxylase] ligase (birA)	36.6%	AF1234	mutator protein MutT (mutT)	63.6%	AF1906	LSU ribosomal protein L18P (rpL18P)	57.8%		
AF1724	dinitrogenase reductase activating glycohydrolase (drgA)	37.9%	AF2200	mutator protein MutT, putative	42.0%	AF1907	LSU ribosomal protein L19E (rpL19E)	55.5%		
AF2232	ferric uptake regulation protein (fur)	25.8%	AF0335	proliferating-cell nuclear antigen (pou30)	33.7%	AF1529	LSU ribosomal protein L21E (rpL21E)	53.2%		
AF1785	iron-dependent repressor	42.0%	AF0694	replication control protein A, putative	30.2%	AF1920	LSU ribosomal protein L22P (rpL22P)	55.2%		
AF2385	iron-dependent repressor	40.0%	AF1024	reverse gyrase (top-RG)	40.7%	AF1923	LSU ribosomal protein L23P (rpL23P)	55.6%		
AF0245	iron-dependent repressor (desR)	28.2%	AF0621	ribonuclease HII (mhb)	39.3%	AF0537	LSU ribosomal protein L24A (rpL24A)	51.4%		
AF1984	iron-dependent repressor (troR)	28.3%	AF1715	type I restriction-modification enzyme, M subunit, authentic frameshift	63.0%	AF0766	LSU ribosomal protein L24E (rpL24E)	61.1%		
AF2430	lacZ expression regulatory protein (lcc)	29.6%	AF1708	type I restriction-modification enzyme, R subunit	38.2%	AF1914	LSU ribosomal protein L24P (rpL24P)	57.8%		
AF1622	leucine responsive regulatory protein (lrp)	29.1%	AF1710	type I restriction-modification enzyme, S subunit	33.0%	AF1918	LSU ribosomal protein L29P (rpL29P)	44.6%		
AF0673	mercuric resistance operon regulatory protein (merR)	37.6%	TRANSCRIPTION			AF1890	LSU ribosomal protein L30E (rpL30E)	41.7%		
AF2425	methanol dehydrogenase regulatory protein (mxoR)	48.3%	DNA-dependent RNA polymerase			AF1904	LSU ribosomal protein L30P (rpL30P)	55.9%		
AF1475	mitochondrial benzodiazepine receptor/sensory transduction protein	38.4%	AF1888	DNA-directed RNA polymerase, subunit A' (rhoA1)	63.6%	AF2066	LSU ribosomal protein L31E (rpL31E)	50.6%		
AF0198	monoamine oxidase regulatory protein, putative	41.9%	AF1889	DNA-directed RNA polymerase, subunit A' (rhoA2)	55.7%	AF1908	LSU ribosomal protein L32E (rpL32E)	51.2%		
AF1933	monoamine oxidase regulatory protein, putative	39.7%	AF1887	DNA-directed RNA polymerase, subunit B' (rhoB1)	65.3%	AF0057	LSU ribosomal protein L37AE (rpL37AE)	47.8%		
AF0978	nitrogen regulatory protein P-II (glnB-1)	61.7%	AF1886	DNA-directed RNA polymerase, subunit B' (rhoB2)	57.1%	AF0874	LSU ribosomal protein L37E (rpL37E)	57.9%		
AF1747	nitrogen regulatory protein P-II (glnB-2)	58.0%	AF2282	DNA-directed RNA polymerase, subunit D' (rhoD)	34.6%	AF2067	LSU ribosomal protein L39E (rpL39E)	56.9%		
AF1750	nitrogen regulatory protein P-II (glnB-3)	60.7%	AF1117	DNA-directed RNA polymerase, subunit E' (rhoE1)	48.4%	AF1430	LSU ribosomal protein L40E (rpL40E)	73.3%		
AF0331	pheromone shutdown protein (traB)	40.5%	AF1166	DNA-directed RNA polymerase, subunit E' (rhoE2)	40.0%	AF1938	LSU ribosomal protein L44E (rpL44E)	46.8%		
AF1797	phosphate regulatory protein, putative	30.7%	AF1885	DNA-directed RNA polymerase, subunit H (rhoH)	59.5%	AF2064	LSU ribosomal protein LXA (rhoLXA)	53.8%		
AF0521	protease synthase and sporulation regulator Pai1, putative	52.4%	AF1131	DNA-directed RNA polymerase, subunit K (rhoK)	61.9%	AF0739	ribosomal protein S18 alanine acetyltransferase	38.5%		
AF1627	repressor protein	59.1%	AF0207	DNA-directed RNA polymerase, subunit L (rhoL)	42.0%	AF2303	ribosomal protein S6 modification protein (rimK)	32.2%		
AF1793	repressor protein	54.5%	AF1130	DNA-directed RNA polymerase, subunit N (rhoN)	58.8%	AF1133	SSU ribosomal protein S2P (rpS2P)	58.3%		
AF0449	response regulator	38.1%	Transcription factors			AF1919	SSU ribosomal protein S3P (rpS3P)	50.0%		
AF1063	response regulator	36.3%	AF1813	TBP-interacting protein TIP49	45.7%	AF1913	SSU ribosomal protein S4E (rpS4E)	48.9%		
AF1256	response regulator	42.5%	AF1299	transcription initiation factor IIB	60.4%	AF2284	SSU ribosomal protein S4P (rpS (			

AF2328	Glu-tRNA amidotransferase, subunit C (gatC)	35.1%	AF1768	protein (dppA)	33.1%	AF2258	multidrug resistance protein	31.3%
AF0815	N ₂ ,N ₂ -dimethylguanosine tRNA methyltransferase (trm1)	38.2%	AF1769	dipeptide ABC transporter, permease protein (dppB)	39.3%	OTHER CATEGORIES		
AF1730	pseudouridylylase synthase I (truA)	37.4%	AF0680	glutamine ABC transporter, permease protein (dppC)	40.8%	<i>Adaptations and atypical conditions</i>		
AF1485	queuine tRNA-ribosyltransferase (tgtB)	44.1%	AF0231	glutamine ABC transporter, periplasmic glutamine-binding protein (glnH)	38.0%	AF0608	ethylene-inducible protein	74.5%
AF0493	ribonuclease PH (rph)	30.8%	AF0232	glutamine ABC transporter, permease protein (glnP)	39.3%	AF0235	heat shock protein (hspK)	32.9%
AF0900	tRNA intron endonuclease (endA)	41.8%	AF0981	osmoprotection protein (proV-1)	39.0%	AF0942	surE stationary-phase survival protein (surE)	50.2%
AF2156	tRNA nucleotidyltransferase (cca)	43.9%	AF0979	osmoprotection protein (proW-1)	32.8%	AF1996	virulence associated protein C (vapC-1)	50.0%
<i>Translation factors</i>			AF0980	osmoprotection protein (proW-2)	36.8%	AF1690	virulence associated protein C (vapC-2)	30.0%
AF2350	ATP-dependent RNA helicase HepA, putative	31.5%	AF0882	osmoprotection protein (proX)	28.7%	<i>Drug and analog sensitivity</i>		
AF2254	ATP-dependent RNA helicase, DEAD-family (deaD)	52.2%	AF0015	proline permease (putP-1)	26.2%	AF1884	daunorubicin resistance ATP-binding protein (drrA)	47.1%
AF0071	ATP-dependent RNA helicase, putative	29.6%	AF0969	proline permease (putP-2)	27.4%	AF1883	daunorubicin resistance membrane protein (drrB)	27.0%
AF1458	ATP-dependent RNA helicase, putative	48.1%	AF1222	proline permease (putP-3)	27.0%	AF0487	penicillin G acylase	31.7%
AF2406	ATP-dependent RNA helicase, putative	35.2%	AF1608	spermidine/putrescine ABC transporter, ATP-binding protein (potA)	50.2%	AF1214	phenylacrylic acid decarboxylase (pad1)	43.2%
AF1149	large helicase-related protein (lhr-1)	34.5%	AF1605	spermidine/putrescine ABC transporter, periplasmic spermidine/putrescine-binding protein (potD), authentic frameshift	31.0%	AF2194	rRNA (adenine-N ₆)-methyltransferase, putative	23.2%
AF2177	large helicase-related protein (lhr-2), authentic frameshift	56.0%	AF1607	spermidine/putrescine ABC transporter, permease protein (potB)	38.0%	AF1696	small multidrug export protein (qacE)	39.0%
AF1220	peptide chain release factor eRF, subunit 1	51.2%	AF1606	spermidine/putrescine ABC transporter, permease protein (potC)	38.7%	<i>Transposon-related functions</i>		
AF2445	SK12-family helicase, authentic frameshift	45.7%	<i>Anions</i>			AF0120	insertion sequence ISH S1, authentic frameshift	34.5%
AF0937	translation elongation factor EF-1, subunit alpha (tuf)	74.4%	AF2308	arsenite transport protein (arsB)	27.3%	AF0193	ISA0963-1, putative transposase, authentic frameshift	34.3%
AF0574	translation elongation factor EF-1, subunit beta	31.3%	AF1415	chloride channel, putative	27.3%	AF0309	ISA0963-2, putative transposase	33.5%
AF1894	translation elongation factor EF-2 (fus)	62.5%	AF0025	cyanoate transport protein (cynX)	24.5%	AF1310	ISA0963-3, putative transposase	33.5%
AF0777	translation initiation factor eIF-1A (eif1A)	57.5%	AF0087	nitrate ABC transporter, ATP-binding protein (nrtC-1)	47.4%	AF1383	ISA0963-4, putative transposase	33.5%
AF0527	translation initiation factor eIF-2, subunit alpha (eif2A)	51.1%	AF0638	nitrate ABC transporter, ATP-binding protein (nrtC-2)	55.5%	AF1410	ISA0963-5, putative transposase	33.5%
AF2326	translation initiation factor eIF-2, subunit beta, putative	45.5%	AF0640	nitrate ABC transporter, ATP-binding protein, putative	32.5%	AF1706	ISA0963-6, putative transposase	33.5%
AF0592	translation initiation factor eIF-2, subunit gamma (eif2G)	64.4%	AF0086	nitrate ABC transporter, permease protein (nrtB-1)	35.4%	AF1836	ISA0963-7, putative transposase, authentic frameshift	23.6%
AF0370	translation initiation factor eIF-2B, subunit delta (eif2BD)	53.3%	AF0639	nitrate ABC transporter, permease protein (nrtB-2)	37.4%	AF0678	ISA1083-1, ISORF2	33.6%
AF2037	translation initiation factor eIF-2B, subunit delta (eif2BD)	57.9%	AF1359	phosphate ABC transporter, ATP-binding protein (pstB)	66.0%	AF0679	ISA1083-1, putative transposase	37.2%
AF0645	translation initiation factor eIF-5A (eif5A)	50.4%	AF1356	phosphate ABC transporter, periplasmic phosphate-binding protein (phoX)	25.1%	AF1351	ISA1083-2, ISORF2	30.8%
AF0768	translation initiation factor IF-2 (infB)	52.2%	AF1358	phosphate ABC transporter, permease protein (pstA)	34.1%	AF1352	ISA1083-2, putative transposase	31.5%
TRANSPORT AND BINDING PROTEINS			AF1357	phosphate ABC transporter, permease protein (pstC)	33.7%	AF2140	ISA1083-3, ISORF2	30.8%
<i>General</i>			AF1360	phosphate ABC transporter, regulatory protein (phoU)	26.9%	AF2139	ISA1083-3, putative transposase	31.5%
AF0393	ABC transporter, ATP-binding protein	34.5%	AF0791	phosphate permease, putative	31.1%	AF0278	ISA1214-1, ISORF2	27.7%
AF0394	ABC transporter, ATP-binding protein	35.2%	AF1798	phosphate permease, putative	52.9%	AF0279	ISA1214-1, putative transposase	33.3%
AF1006	ABC transporter, ATP-binding protein	35.1%	AF0092	sulfate ABC transporter, ATP-binding protein (cysA)	54.2%	AF0305	ISA1214-2, ISORF2	27.7%
AF1018	ABC transporter, ATP-binding protein	57.7%	AF0093	sulfate ABC transporter, permease protein (cysT)	44.1%	AF0306	ISA1214-2, putative transposase	33.3%
AF1021	ABC transporter, ATP-binding protein	37.8%	<i>Carbohydrates, organic alcohols, and acids</i>			AF0642	ISA1214-3, ISORF2	26.5%
AF1136	ABC transporter, ATP-binding protein	39.3%	AF0347	C4-dicarboxylate transporter (mae1)	24.5%	AF0857	ISA1214-4, ISORF2	27.7%
AF1139	ABC transporter, ATP-binding protein	38.2%	AF1426	glycerol uptake facilitator, MIP channel (glpF)	36.2%	AF0858	ISA1214-4, putative transposase	33.3%
AF1300	ABC transporter, ATP-binding protein	34.1%	AF0013	hexuronate transporter (exuT)	25.1%	AF2091	ISA1214-5, ISORF2	26.5%
AF1469	ABC transporter, ATP-binding protein	43.5%	AF0806	L-lactate permease (lcpP)	31.7%	AF2092	ISA1214-5, putative transposase	33.3%
AF1819	ABC transporter, ATP-binding protein	51.1%	AF0008	oxalate/formate antiporter (oxfT-1)	25.7%	AF2223	ISA1214-6, ISORF2	26.5%
AF1982	ABC transporter, ATP-binding protein	41.3%	AF0367	oxalate/formate antiporter (oxfT-2)	33.2%	AF2222	ISA1214-6, putative transposase	25.6%
AF2364	ABC transporter, ATP-binding protein	53.5%	AF1069	pantothenate permease (panF-1)	28.9%	AF0138	transposase IS240-A	43.3%
AF1005	ABC transporter, ATP-binding protein, putative	28.7%	AF1205	pantothenate permease (panF-2)	24.8%	AF0895	transposase IS240-A	46.2%
AF1064	ABC transporter, ATP-binding protein, putative	36.0%	AF0237	pantothenate permease (panF-3)	25.1%	AF2390	transposase, authentic frameshift	24.0%
AF1983	ABC transporter, periplasmic binding protein	25.4%	AF0041	polysaccharide ABC transporter, ATP-binding protein (rtbB-1)	42.5%	AF0137	transposase, putative	29.6%
AF1981	ABC transporter, permease protein	29.9%	AF0290	polysaccharide ABC transporter, ATP-binding protein (rtbB-2)	43.9%	AF1628	transposase, putative	32.8%
AF1995	sodium- and chloride-dependent transporter	52.5%	AF0042	polysaccharide ABC transporter, permease protein (rtbA-1)	27.5%	UNKNOWN		
<i>Amino acids, peptides and amines</i>			AF0289	polysaccharide ABC transporter, permease protein (rtbA-2)	28.5%	AF0477	AAA superfamily ATPase	35.0%
AF1766	amino acid ABC transporter, periplasmic binding protein/protein kinase	27.4%	AF0887	ribose ABC transporter, ATP-binding protein (rbsA-1)	33.3%	AF0513	allene oxide synthase, putative	39.5%
AF0222	branched-chain amino acid ABC transporter, ATP-binding protein (braF-1)	42.7%	AF1170	ribose ABC transporter, ATP-binding protein (rbsA-2)	27.9%	AF0478	ATP-binding protein PhnP (phnP)	30.9%
AF0822	branched-chain amino acid ABC transporter, ATP-binding protein (braF-2)	44.7%	AF0888	ribose ABC transporter, permease protein (rbsC-1)	24.1%	AF1775	atrazine chlorohydrolase, putative	34.4%
AF0959	branched-chain amino acid ABC transporter, ATP-binding protein (braF-3)	37.6%	AF0889	ribose ABC transporter, permease protein (rbsC-2)	31.2%	AF0973	bile acid-inducible operon protein F (bafF-1)	30.8%
AF1390	branched-chain amino acid ABC transporter, ATP-binding protein (braF-4)	59.7%	AF2014	sugar transporter, putative	26.0%	AF0974	bile acid-inducible operon protein F (bafF-2)	29.9%
AF0221	branched-chain amino acid ABC transporter, ATP-binding protein (braG-1)	48.2%	<i>Cations</i>			AF1315	bile acid-inducible operon protein F (bafF-3)	31.3%
AF0823	branched-chain amino acid ABC transporter, ATP-binding protein (braG-2)	42.9%	AF3977	ammonium transporter (amt-1)	44.3%	AF2063	c-myc binding protein, putative	21.7%
AF0958	branched-chain amino acid ABC transporter, ATP-binding protein (braG-3)	34.1%	AF1746	ammonium transporter (amt-2)	40.0%	AF1992	calcium-binding protein, putative	31.2%
AF1389	branched-chain amino acid ABC transporter, ATP-binding protein (braG-4)	64.6%	AF0473	cation-transporting ATPase, P-type (pacS)	44.0%	AF2287	carotenoid biosynthetic gene ERWORTS, putative	49.4%
AF0223	branched-chain amino acid ABC transporter, periplasmic binding protein (braC-1)	34.3%	AF0152	copper-transporting ATPase, P-type (copB)	44.5%	AF0512	chloroplast inner envelope membrane protein	42.5%
AF0827	branched-chain amino acid ABC transporter, periplasmic binding protein (braC-2)	26.8%	AF2394	iron (II) transporter (feoB-1)	33.3%	AF2251	competence-damage protein, putative	28.0%
AF0962	branched-chain amino acid ABC transporter, periplasmic binding protein (braC-3)	25.6%	AF0561	iron (II) transporter (feoB-2)	48.0%	AF0090	dehydrogenase	34.1%
AF1391	branched-chain amino acid ABC transporter, periplasmic binding protein (braC-4)	50.1%	AF0430	iron (III) ABC transporter, ATP-binding protein (hemV-1)	50.4%	AF1498	dehydrogenase, putative	29.4%
AF0224	branched-chain amino acid ABC transporter, permease protein (braD-1)	25.4%	AF1401	iron (III) ABC transporter, ATP-binding protein (hemV-2)	58.7%	AF1518	DNA/pantothenate metabolism flavoprotein, putative	51.4%
AF0825	branched-chain amino acid ABC transporter, permease protein (braD-2)	30.8%	AF1397	iron (III) ABC transporter, ATP-binding protein (hemV-3)	35.2%	AF0039	dolichol-P-glucose synthetase, putative	33.7%
AF0961	branched-chain amino acid ABC transporter, permease protein (braD-3)	23.9%	AF0432	iron (III) ABC transporter, ATP-binding protein (hemV-4)	58.7%	AF0328	dolichol-P-glucose synthetase, putative	39.0%
AF1392	branched-chain amino acid ABC transporter, permease protein (braD-4)	65.4%	AF1402	iron (III) ABC transporter, permease protein (hemU-1)	36.2%	AF0581	dolichol-P-glucose synthetase, putative	27.5%
AF0225	branched-chain amino acid ABC transporter, permease protein (braE-1)	28.7%	AF0786	iron (III) ABC transporter, permease protein (hemU-2)	36.2%	AF0569	DR-beta chain MHC class II	37.7%
AF0824	branched-chain amino acid ABC transporter, permease protein (braE-2)	31.3%	AF0346	magnesium and cobalt transporter (corA)	40.1%	AF0383	endonuclease III, putative	47.1%
AF0960	branched-chain amino acid ABC transporter, permease protein (braE-3)	30.1%	AF0866	mercuric transport protein periplasmic component (merP)	35.2%	AF1150	erpK protein, putative	54.9%
AF1393	branched-chain amino acid ABC transporter, permease protein (braE-4)	60.5%	AF0217	Na ⁺ /H ⁺ antiporter (napA-1)	28.2%	AF2372	extragenic suppressor (subB)	37.0%
AF1612	cationic amino acid transporter (cat-1)	29.5%	AF1245	Na ⁺ /H ⁺ antiporter (napA-2)	28.4%	AF1418	glycerol-3-phosphate cytidyltransferase (taqD)	56.6%
AF1774	cationic amino acid transporter (cat-2)	38.0%	AF0846	Na ⁺ /H ⁺ antiporter (rhe2)	33.1%	AF0744	GTP-binding protein	33.4%
AF1770	dipeptide ABC transporter, ATP-binding protein (dppD)	47.8%	AF0715	potassium channel, putative	39.5%	AF1181	GTP-binding protein	36.3%
AF1771	dipeptide ABC transporter, ATP-binding protein (dppF)	43.1%	AF1673	potassium channel, putative	36.3%	AF1364	GTP-binding protein	57.5%
AF1767	dipeptide ABC transporter, dipeptide-binding		AF2197	potassium uptake system protein (trkA-1)	24.6%	AF2146	GTP-binding protein	65.9%
			AF0838	TRK potassium uptake system protein (trkA-2)	42.9%	AF0428	GTP-binding protein, GTP1/OBG-family	43.9%
			AF0839	TRK potassium uptake system protein (trkH)	39.8%	AF2237	HAM1 protein	31.4%
			<i>Other</i>			AF2211	HIT family protein (hit)	29.6%
			AF0834	ferritin, putative	39.8%	AF0216	L-isospartyl protein carboxyl methyltransferase	
			AF1980	heme exporter protein C (helC)	29.0%		PimT, putative	35.5%
			AF1144	multidrug resistance protein	29.2%	AF2313	maoC protein (maoC)	43.0%
			AF1325	multidrug resistance protein	29.9%	AF0429	methyltransferase	43.8%
						AF0186	nifS protein, class-V aminotransferase (nifS-1)	46.1%
						AF0564	nifS protein, class-V aminotransferase (nifS-2)	46.1%
						AF0185	nifU protein (nifU-1)	55.6%
						AF0565	nifU protein (nifU-2)	55.6%
						AF0632	nifU protein (nifU-3)	47.4%
						AF1781	nodulation protein NteD (nfeD)	33.4%
						AF2269	nucleotide-binding protein (spvG)	48.7%
						AF2382	nucleotide-binding protein	49.1%
						AF0374	p-nitrophenyl phosphatase (pho2)	31.7%
						AF1978	periplasmic divalent cation tolerance protein (cutA)	31.3%
						AF1652	prepro-subtilisin sendai, putative	31.6%
						AF2021	rod shape-determining protein (mreB)	26.6%
						AF1778	stage V sporulation protein (spvG)	43.9%
						AF1970	TPR domain-containing protein	29.0%
						AF2202	tryptophan-specific permease, putative	25.2%
						AF0816	vfp-therm, putative	42.1%
						AF1679	vfp-therm, putative	45.1%

Structure and function of the vertebrate magnetic sense

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Some vertebrates can navigate over long distances using the Earth's magnetic field, but the sensory system that they use to do so has remained a mystery. Here we describe the key components of a magnetic sense underpinning this navigational ability in a single species, the rainbow trout (*Oncorhynchus mykiss*). We report behavioural and electrophysiological responses to magnetic fields and identify an area in the nose of the trout where candidate magnetoreceptor cells are located. We have tracked the sensory pathway from these newly identified candidate magnetoreceptor cells to the brain and associated the system with a learned response to magnetic fields.

The use of the Earth's magnetic field by animals for navigation during migration and homing has been studied intensively¹ but the detection mechanism and the sensory pathway between the detector and the brain that underpin navigation behaviour remain unknown. Hypotheses have been proposed that link the detection of magnetic fields to vision, electroreception and magnetite particles^{2–6}. Magnetic detection mechanisms mediated through the visual system^{2,3} seem to be contradicted by the ability of salmon, turtles and lobsters to orientate magnetically in total darkness^{7–9}. Similarly, electrical induction could mediate magnetoreception by elasmobranchs⁴ but not by species that do not detect electrical fields. Magnetoreception using magnetite particles^{5,6} could readily permit the known responses to magnetic field stimuli^{10,11} and is supported by impairment¹² and pulse magnetization^{13,14} experiments. But magnetite-based magnetoreceptors have not yet been found in any animal. Thus, each of these detection mechanisms has so far failed at some level to provide a convincing description of how animals detect magnetic fields.

Identifying and describing the components of the magnetic sensory system presents several challenges. First, magnetic fields are relatively simple stimuli with only two dimensions—direction and intensity—which vary slowly in space and time. Hence, large numbers of sensory cells, corresponding large numbers of sensory nerves and complex central processing centres are not necessary for magnetic field detection. Second, animals only respond to magnetic fields if they are actively moving or attempting to move¹⁵, which makes it difficult to elicit powerful behavioural responses to magnetic fields in most laboratory situations. Third, tissues are transparent to magnetic fields, making accessory sensory structures such as lenses that focus the stimuli on receptor cells unnecessary. Receptor cells could be dispersed throughout an organism. Finally, single-domain magnetite particles suitable for use in magnetoreception¹⁰ would be too small (≤ 100 nm) for detection by conventional light microscopy and too rare (≤ 5 p.p.b. by volume) for detection by conventional transmission electron microscopy (TEM). It should therefore come as no surprise that standard behavioural, physiological and anatomical approaches to the study of sensory systems have been difficult to apply to the magnetic sense.

We have now recorded behavioural and electrophysiological responses to magnetic fields and identified candidate receptor cells in the trout. We have used a discrimination training

procedure¹⁶ to demonstrate robust behavioural responses by the trout to the presence and absence of a magnetic field anomaly in experimental tanks. We have identified single neurons in the superficial ophthalmic ramus (ros V) of the trigeminal nerve that respond to changes in the intensity but not the direction of an imposed magnetic field, and used a combination of new imaging and microscopic techniques to identify candidate magnetite-based magnetoreceptor cells in the nose of the trout. The cells contain crystals that are iron-rich and almost identical in form to single-domain magnetite crystals previously extracted from sockeye salmon¹⁷. We have also traced fine branches of the ros V nerve to the same cellular layer (the lamina propria within the olfactory lamellae) as the candidate magnetoreceptor cells. Identification of the critical components of the magnetic sense brings a new perspective to the study of long-distance orientation in a variety of vertebrate groups.

Learned magnetic discrimination

Our experiments exploited previous findings that magnetic discrimination training is successful when the subjects must move to sample spatial patterns in intensity of the magnetic field^{18–20} but fails when the experimental stimuli differ only in direction^{1,18,21,22}. We trained three juvenile rainbow trout individually to swim to and strike repeatedly at a target to obtain food (Fig. 1a). Our measure of magnetic stimulus discrimination was therefore the rate at which the fish struck the target in anticipation of reinforcement with food or lack of reinforcement at the end of each trial¹⁶. The magnetic stimuli to be discriminated were the presence and absence of a magnetic intensity anomaly focused on the target and superimposed on the background of the Earth's magnetic field.

A clear difference in response rates developed after the third training session (Fig. 1b) with the fish responding at a consistently higher rate to the reinforced stimulus (S+) than to the non-reinforced stimulus (S–). The difference between the average response rates to S+ and S– was statistically reliable (ANOVA: $F_{1,2}$ stimuli = 25.9281, $P = 0.0365$). The response to S– was more variable in individual fish than the response to S+ ($F_{2,4}$ subjects = 19.2265, $P = 0.0086$). There seemed to be no difference if the magnetic anomaly was the reinforced or non-reinforced stimulus as the graphs for individual fish were all similar to Fig. 1b. The separation of response rates to S+ and S– with time during the experiments was confirmed statistically ($F_{14,192}$ subjects $\times$ stimuli $\times$ sessions = 9.64375, $P = 0.0403$). The likely cause of this effect is an interaction between the development of discrimination (response rates to S+ and S– changed with time

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during the experiment) and variability of behaviour among the subjects.

Our results are consistent with the idea that animals learn most readily to discriminate magnetic fields when the conditioned response requires movement and when the fields are spatially distinctive. Yellowfin tuna, honeybees and now rainbow trout all readily discriminated magnetic fields under these conditions but the tuna and honeybees both failed to discriminate when one of these conditions was not met^{18,20}. Despite these constraints, however, magnetic discrimination by the trout can now be analysed in the same ways as discrimination of other stimuli by other species (for example, ref. 16).

Neural responses to magnetic field intensity

We recorded from sensory nerves and areas of the brain that receive neural input from the anterior skull, where magnetite had previously been found^{17,23}. The only positive responses were found in the ros V nerve. The nerve runs in close association with the superficial ophthalmic ramus of the dorsal anterior lateral-line nerve (ros1) above the eye. The two rami form the supra-orbital trunk (SOT; ref. 24). These rami separate after passing through the orbit and innervate the snout area of the head.

We used a series of search stimuli presented as uniform square waves that changed direction, intensity, or both direction and intensity of the magnetic field (represented by B in Fig. 2) around the head of experimental fish. The stimuli were produced by subtracting, adding and switching between subtracting and adding 50 microTesla (μT) to the background field ($25\mu\text{T}$; see Methods) in the experimental tank. For the first search stimulus (designated SS1), subtracting $50\mu\text{T}$ reversed the magnetic field direction in the tank (represented by $B:-B$ for onsets and $-B:B$ for offsets in Fig. 2) but did not change intensity. In contrast, adding $50\mu\text{T}$ (SS2) trebled the intensity without changing field direction in the tank (represented by $B:3B$ for onsets, $3B:B$ for offsets in Fig. 2). Our third stimulus (SS3) combined these stimuli by reversing the polarity of the input current to the coils rather than switching it off. The field in the situation was thus switched between a field with the same intensity but reversed direction relative to the background field and a field with the same direction but three times the intensity of the background field ($-B:3B$ for onsets; $3B:-B$ for offsets in Fig. 2).

The square-wave stimuli induced transient voltage-gradient artefacts of 1.4 V cm^{-1} in 50% sea water that could potentially affect the activity of the nerves being recorded. The artefacts were, however, mirror images of each other when current polarities were reversed. That is, the artefact caused by the onset of SS2 was identical to the artefact caused by the offset of SS1, and vice versa. Response to the onset, for example, of SS2 but not to the offset of SS1 would therefore distinguish between response to the magnetic stimulus and response to the associated artefact.

We located two populations of spontaneously active neurons in the ros V nerve (Fig. 2a). The 'fast' population showed high-amplitude signals ($>7.5\text{ mV}$ above background in extracellular recordings) and high firing rates (mean 39 Hz). The 'slow' population showed low-amplitude signals ($1\text{--}2\text{ mV}$ above background) and low firing rates (mean 20 Hz) that were significantly lower than for the fast units ($t_s = 3.2$, $P < 0.01$ for the firing rates of the units shown in Fig. 2a). None of the fast units ever responded to magnetic stimuli, whereas a small proportion of the slower units responded to magnetic stimuli with excitatory responses to the onset of SS2 (Fig. 2c–e) as well as both excitatory and inhibitory responses to the onsets and offsets of SS3 (Fig. 2f).

The responsive units showed regular firing patterns, as seen by the normally distributed intervals between spikes (Fig. 2b), except during the transient responses to SS2 and SS3. To our surprise, no unit responded to SS1, nor was there any off-response corresponding to the excitatory on-responses to SS2 and SS3 (Fig. 2d). Response to SS2 presented at 0.5 and 1 Hz by two of the units is

shown in Fig. 2e. The latency and time-course (the first point after the stimulus step and the period during which the firing rate was more than two standard deviations above the mean for each unit) of the responses by the two units exposed to both stimulation frequencies were similar but the peak amplitudes of the responses increased and decreased, respectively, when the rate at which SS2 was presented increased from 0.5 to 1 Hz (Fig. 2e).

Five units responded to SS3 (of 43 slow units tested with this stimulus). Three units responded to the step where intensity increased from $-B$ to $3B$ and one responded to the step where the intensity decreased from $3B$ to $-B$ (Fig. 2f). Both response latencies and durations for these units ranged between 15 and 80 ms, with peak amplitudes of response ranging between 4.8 and 16.8 times the background firing rate for units exhibiting excitation. The firing rate of the fifth unit that responded to SS3 was nearly halved for about 40 ms after the onset of SS3 (mean $\pm$ s.d. firing rates in post-stimulus time histograms: 4–42 ms after stimulus onset, $25.2 \pm 17.4\text{ Hz}$; 43–442 ms after stimulus onset, $36.3 \pm 25.0\text{ Hz}$; $t_s = 2.7$, $P < 0.01$).

Although the responses by units in the ros V to magnetic field intensity are consistent with the behavioural responses in the trout, it is important to exclude other explanations for our electrophysiological data. The failure of the units in the ros V that responded to SS2 to respond to SS1 ($B:-B$ and $-B:B$ in Fig. 2d) counters the possible arguments that the units responded either to changes in magnetic field direction that were too small for us to detect or to electrical or mechanical artefacts induced by SS2 ($B:3B$ and $3B:B$)

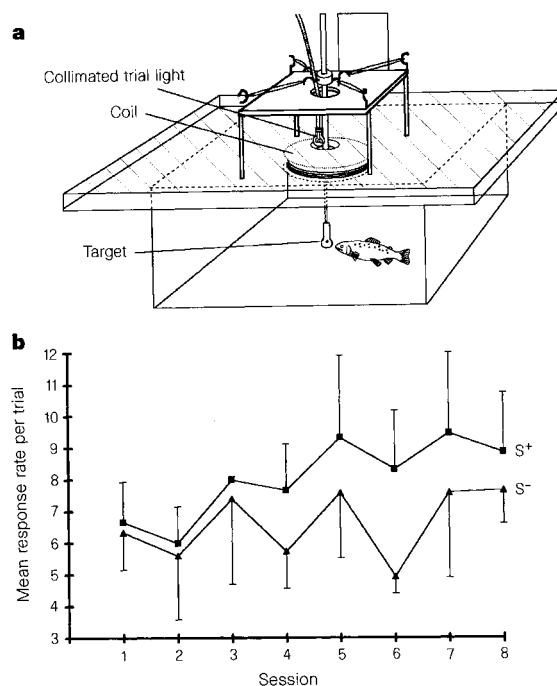


Figure 1 Discrimination of the presence and absence of a magnetic anomaly by rainbow trout. **a**, A 50-turn coil (4 cm diameter) induced a narrowly localized magnetic field anomaly (peak intensity $70\mu\text{T}$) which, when added to the local background field ($55\mu\text{T}$), produced a peak intensity of $125\mu\text{T}$ at the water surface directly below the axis of the coil. A response bar, tipped with a teflon target and aligned with the axis of the coil, projected just below the water surface at the centre of the tank. Liquid fish-food pumped through the bar and delivered at the target permitted a close association of stimulation, response and reinforcement. The experimental procedures were fully automated. **b**, Magnetic discrimination data presented as the mean response rate per trial by the three fish with the data blocked over the five S+ and S- trials given in balanced quasi-random order in each training session. Vertical lines indicate s.e.m.

because all such artefacts were equivalent in both search stimuli. Conversely, the failure of the units that responded to SS2 to respond to SS1 does not rule out the existence of units that would respond to SS1.

Candidate magnetoreceptor cells

Demonstrating a magnetite-based detection mechanism depends first on the identification of magnetite associated with candidate magnetoreceptor cells. Detecting single-domain magnetite *in situ* is difficult because of the small size of the crystals and their very low volume concentrations in tissues. We have partly resolved the detection problem in large volumes of tissue by using the confocal laser scanning microscope (CLSM) in reflection mode to render chains of single-domain magnetite visible at the light microscope level. The chains of magnetite discovered in magnetotactic bacteria²⁵ reflect the laser light to produce bright spots which are clearly associated with the bacterial cells when viewed in transmitted light (Fig. 3a). A search through rainbow trout heads embedded in both JB4 and Epon resins using CLSM revealed crystalline material in the lamina propria layer within olfactory lamellae. These crystals gave reflections of similar intensity and dimensions to the magnetite in the bacteria (Fig. 3b). The lamina propria is the layer separating the columnar epithelial folds that make up each lamella. It is separated from the olfactory epithelial layer by a basal lamina. Serial thin sections through one of the reflections in the lamina propria revealed the crystal (arrows) shown in Fig. 3c in bright-field (left) and dark-field (centre) TEM. Further TEM analysis showed that the

crystal was iron-rich (Fig. 3d) and almost identical in size (length 50 nm) and shape to single-domain magnetite extracted from sockeye salmon¹⁷.

The cells containing the reflecting particles are 10–12 μm in length, have a distinctive tri-lobed shape and are consistently located near the basal lamina of the olfactory epithelium. Cells containing reflections are relatively rare, with never more than two or three being detected at a time within the volume (roughly 150 μm^3) sampled by the CLSM at the magnification required to detect the reflections. In addition, the spots produced by the crystals in the olfactory epithelium were punctate in both the horizontal and vertical dimensions. We detected reflections in other regions of the trout head but these could be excluded as possible sites for the magnetoreceptors. Reflections associated with bone in the head of the trout were easily detectable at low magnification in CLSM and differed in appearance and dimensions from the reflections produced by magnetite in the bacteria. In contrast, reflections from the pigment melanin could be excluded on the basis of their clear association with pigment cells, which are large and have a distinctive stellate morphology.

The similarity of the reflectances in the trout to magnetite reflectances in the bacteria, and their consistent location near the basal lamina of the olfactory epithelium of the trout, lead us to view the reflectance-containing cells as candidate magnetoreceptors. We are encouraged that the crystal shown in Fig. 3 was similar in size, shape and presence of iron to magnetite extracted from sockeye salmon¹⁷. The crystal is likely to be part of a larger group such as a

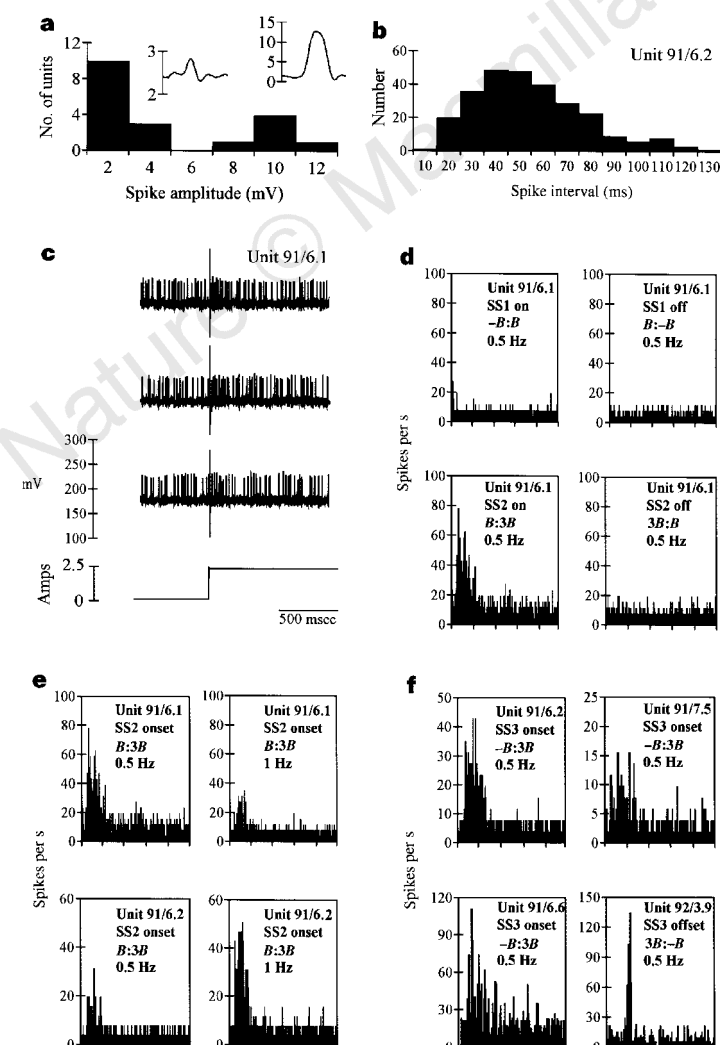


Figure 2 Electrophysiological responses to magnetic fields. **a**, Histogram of spike amplitudes for 19 units recorded as they were encountered in the ros V of the rainbow trout. We divided the units recorded into two populations on the basis of spike amplitudes (illustrated in the inset plots of single spikes representing each population). Mean firing rates $\pm$ s.d. for the two populations of units were 20 ± 10.3 Hz and 39 ± 14.9 Hz. Responsive units were found only in the population of units with low spike amplitudes and firing rates. Ordinates in mV for inset plots of single spikes. **b**, Histogram of spike intervals in the absence of experimental stimulation for the unit 91/6.2 (one of the responsive units shown in **e**; 286 spike intervals recorded). The median spike interval for the unit is 45 ms. The data are roughly normally distributed, consistent with a regular firing rate of about 20 Hz. **c**, Peristimulus activity of a single unit (91/6.1) in the ros V of the rainbow trout. The onset of SS2 (bottom trace) is aligned with the associated stimulus artefacts in the top three traces (clipped for clarity). To the left of the artefacts, the unit is spontaneously active in the background magnetic field. To the right of the artefacts, the traces show the activity of the unit for 1 s after the onset of SS2. Acceleration of the firing rate of the unit is evident for the first 100 ms after the stimulus step. **d**, Post-stimulus time histograms (PSTHs) of responses by the unit shown in **c** to SS1 and SS2 presented 128 times at 0.5 Hz (on for 1 s then off for 1 s). **e**, PSTHs of responses by two spontaneously active units to the onsets of SS2 presented 128 times at 0.5 and 1 Hz. **f**, PSTHs of responses by four spontaneously active units to SS3. The stimuli were presented 128 times in each case. Each plot in **d–f** begins at the step change in the field and is of duration 500 ms. The magnetic field remained constant throughout the period. Unit identification number, search stimulus number, stimulus step and presentation rates are listed. Sampling bin widths are 2 ms in **d**, **e** and in the upper-left panel of **f** and 4 ms in the remaining panels of **f**. Tick marks on the abscissae are at 100 ms intervals.

chain of crystals, as is the case in the magnetotactic bacteria, because it was too small on its own to produce a reflection that could be detected at the light microscope level.

Association between magnetoreceptors and nerves

If the cells in the lamina propria of the olfactory lamellae do indeed contain magnetite used in magnetoreception, it is reasonable to predict that the magnetically responsive nerve should be linked to the candidate receptor cells. We have used serial histological sections and DiI, a fluorescent lipophilic dye, to trace branches of the ros V from the site where electrophysiological recordings of responses to magnetic field stimulation were made, to the endings of the individual nerve cells (Figs 4–6).

DiI migrated in both anterograde and retrograde directions along myelinated and unmyelinated fibres in the ros V. Posterior to the orbit, it joined other branches of the trigeminal nerve and ended in cell bodies that make up part of the anterior ganglion. From the ganglion, the labelled nerve tracts entered the anterior dorsal area of the medulla oblongata. Anterior to the orbit, the ros V coalesces with the ros I as it passes through a foramen of the neurocranium to the snout region. After leaving the foramen, the ros V separates

again from the ros I and has rami that surround the olfactory nerve, innervate the superficial epidermis of the snout and surround, as well as penetrate, the nasal capsule.

The ros V rami that surround the nasal capsule form a complex network from which very fine processes penetrate the capsule wall and terminate in the lamina propria of the olfactory lamellae. To link these fine processes back to the network, we developed a combination of techniques using CLSM optical slicing, computerized three-dimensional image-stack analysis and reconstruction,

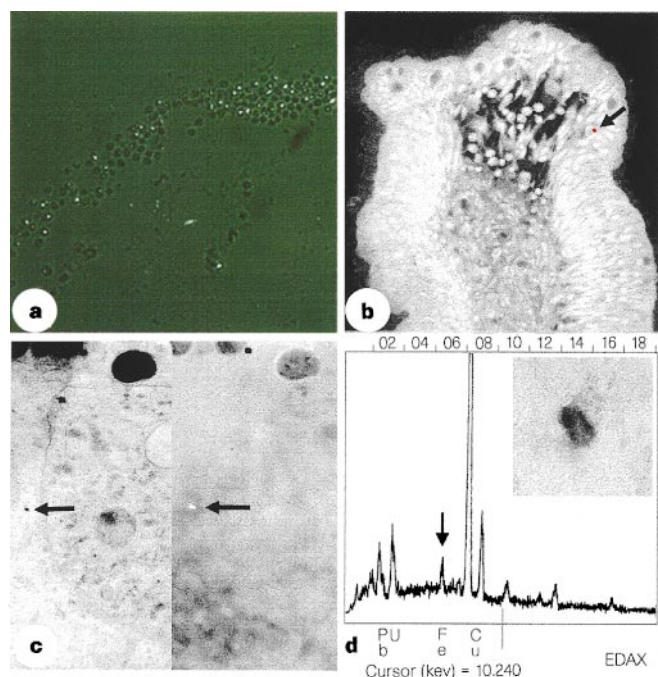


Figure 3 Detection of intracellular magnetite. **a**, Overlaid images of magnetotactic bacteria collected using the method of ref. 37, dried onto a coverslip and viewed using a CLSM in reflected and transmitted light modes. Bright spots observed in reflection mode coincide with the location of the magnetotactic bacteria cells viewed in transmitted light mode (magnification $\times 290$). **b**, A reflectance contained in a cell located near the basal lamina of the olfactory epithelium of the trout has been false-coloured red (arrow) and overlaid onto an autofluorescence image of the olfactory lamella taken at the same depth and magnification ($\times 190$). The lamina propria is the central space that separates the folds of the columnar olfactory mucosa. **c**, Bright-field (left) and dark-field (right) TEM of a crystal associated with a reflectance in the trout olfactory lamellae. In bright-field TEM, both the crystal (arrow) and a much larger pigment granule (top centre) are electron-dense. In dark-field TEM, the crystal (arrow) reflects the electron beam strongly whereas the large pigment granule (upper right) does not (magnification $\times 12,500$). **d**, Energy dispersive analysis of X-ray emissions (EDAX) of the crystal in **c**. Inset shows the crystal (length 50 nm) at higher magnification. The copper (Cu) peak is due to the copper grid used, and lead (Pb) and uranium (U) peaks are from TEM stains. The peak from iron (Fe) present in the crystal is indicated by an arrow. This peak was absent in control regions of the same section.

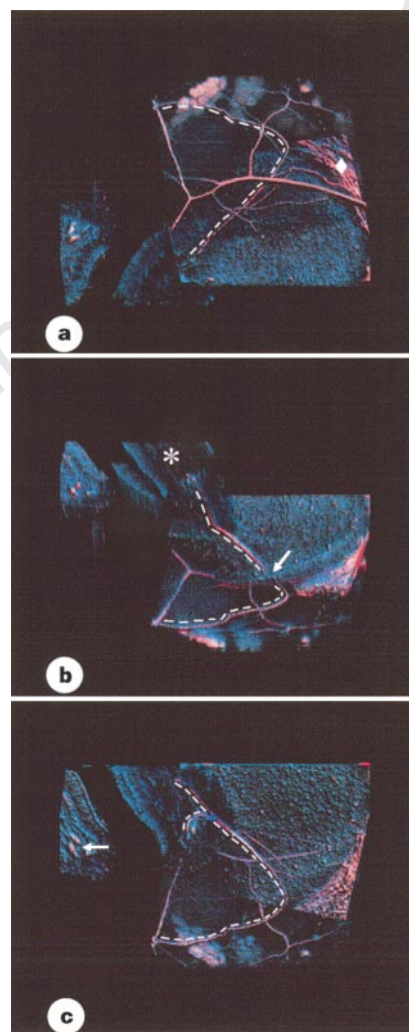


Figure 4 Three steps in a 270° rotation series of a three-dimensional volume-rendered image stack showing the path of a nerve process (right of dashed line) as it enters the top of a lamella. Lipid deposits can be seen on the outside of the nasal membrane (top centre of **a** and bottom centre of the rotated volumes **b** and **c**, and correspond to the yellow layer of Fig. 6c). **a**, The rendered volume rotated 70° from the top shows part of a fine process (right of dashed line) of the ros V as it goes over the outside of the nasal capsule and curves downwards and through the nasal membrane. A representative region of the extensive network that surrounds the outside of the nasal capsule can be best seen to the right of the image (indicated by diamond symbol). **b**, The rendered volume vertically rotated 162° from **a** to show the nasal capsule from within, and the fine process penetrating through the nasal membrane (arrow) from the outside and continuing into a lamella (asterisk). **c**, The rendered volume has been rotated a further 35° and the fine process can be seen to branch within the lamella, one branch curving around the lamellar artery and the other proceeding along towards the lamellar base. The artery is 'U'-shaped at the top of the lamella; its ascending and descending arms are more clearly seen in the lamina propria layer of the lamella on the left (arrow) (magnification $\times 65$).

and volume rotations. After analysing the images individually, we reconstructed areas of the nasal capsule in three dimensions, allowing us to rotate the volumes and definitively trace the fine processes within the lamina propria back to the specific rami of the ros V that surround the nasal capsule (Figs 4, 6).

From these reconstructions, we identified two distinct branching patterns by which the ros V innervates the olfactory lamellae. The first of these was a series of nerves that penetrated the capsule wall individually and entered a lamella from the top (Figs 5a–c, 6), dividing at least once within the area of the lamina propria (Fig. 5c) before terminating in finer processes. These fine processes reached the distal portions of the olfactory lamellae, where the candidate magnetoreceptor cells were most often found. One of these fine processes within the lamina propria consistently wrapped around the artery that loops through each olfactory lamella (Fig. 4c). The second branching pattern appeared to arise from a nerve that penetrated the capsule from the base. Successive CLSM optical slices showed that this nerve branched and penetrated several olfactory lamellae from their bases (Fig. 5d). None of the fine processes from either pattern were seen to penetrate the basal lamina or enter the olfactory mucosal layer itself. This dual innervation pattern was subsequently traced in five separate fish.

We can now propose a link from the candidate magnetoreceptor cells in the lamina propria of the olfactory lamellae through the ros V to the brain, summarized in Fig. 6. Our work also revealed a complex and previously undescribed network of ros V fine processes around the olfactory capsule. The function of this network is unknown, but its size, complexity and innervation of the olfactory lamellae invite further investigation.

Implications for magnetoreception

We suggest that vertebrates detect magnetic fields using magnetite-based magnetoreceptors located in the lamina propria of the olfactory epithelium and linked to the brain via the ros V. The presence of both behavioural and electrophysiological responses to magnetic intensity in the trout and the close association between the magnetically responsive nerve and the candidate magnetoreceptor cells indicate that the responses and the candidate receptors are

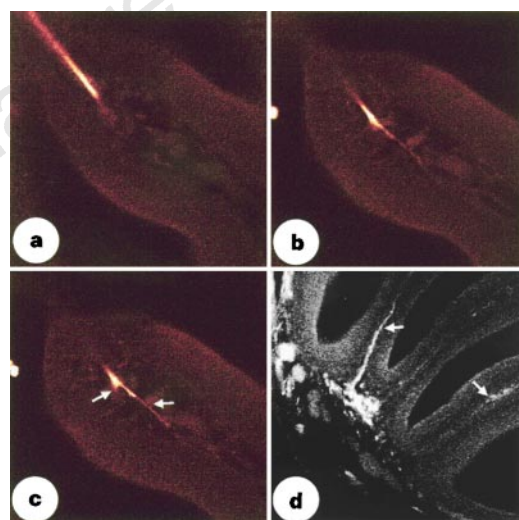


Figure 5 Optical slices showing two different branching patterns of Dil-labelled nerve processes entering trout olfactory lamellae. **a–c**, Three optical slices through a single olfactory lamella (magnification $\times 135$). A labelled fine process from a branch of the ros V can be seen entering the lamella through the top (**a**) and forming two fine processes (in **b**, 22 μm from **a** and in **c**, 27 μm from **a**; arrows). **d**, Fine processes can also be seen entering the lamina propria of several lamellae (arrows) from their bases (magnification $\times 55$). These processes originate from a different branch of the ros V than the one that innervates the top area in **a–c**.

likely to be functionally linked. Robust responses to changes in magnetic field intensity have now been demonstrated behaviourally in fish¹⁸ and turtles⁷, and electrophysiologically in the ros V of fish and in the trigeminal system of birds²⁶. Our reconstructions clearly show that fine processes of the ros V terminate in the region (within the lamina propria of the olfactory lamellae) where the candidate magnetoreceptor cells are found (Fig. 6). Abolition of the effects of impulse magnetization on magnetic orientation behaviour^{13,14} by pharmacological blocking of the trigeminal nerve system²⁷ in birds provides complementary evidence linking behavioural responses to candidate magnetite-based magnetoreceptors. Our results thus

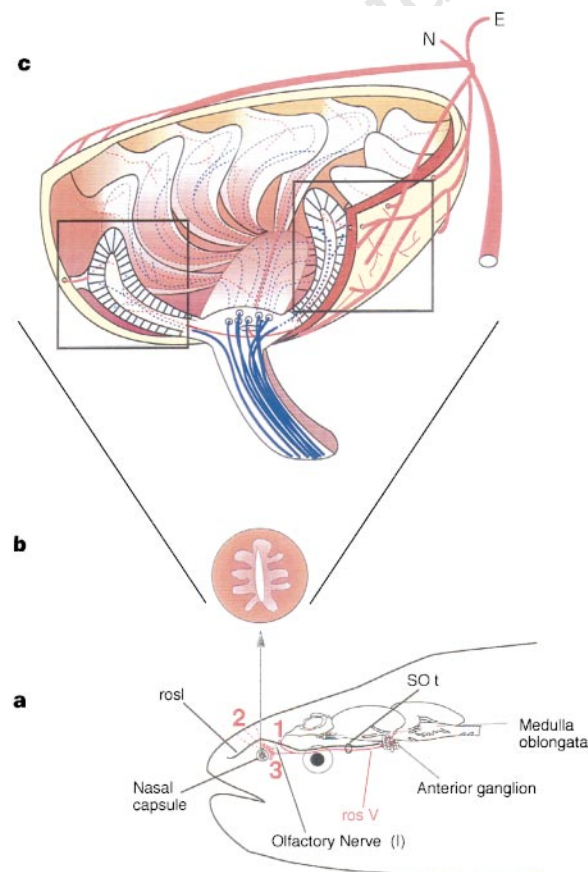


Figure 6 Schematic diagram of the innervation of the head region and nasal capsule of the trout by the SOT branch of the ros V. **a**, Innervation of the SOT (the ros V is shown in red) in the head region of the trout. **b**, Olfactory rosette within the trout nasal capsule (top view). The nasal flap that lies over the top of the olfactory rosette has been removed for clarity. **c**, A three-dimensional diagram of the innervation by the ros V into olfactory lamellae in the nasal capsule of the trout. One process innervates the nasal membrane and flap (N) and the other (top right (E)) is process 2 in **a**. Others form a network of nerves which surround the nasal capsule. Within this network, the smaller branches have fine processes that pass through the nasal membrane lining the nasal capsule and innervate, at both the top and base, individual olfactory lamellae that form the olfactory rosette. The olfactory nerve (blue) is the combination of all axons of the olfactory sensory cells which are situated in the mucosa and send their axons to the olfactory bulb. The network of nerves surrounding the capsule generally lies in a fatty layer (yellow) that is typically found between the neurocranium (not shown) and the outer membrane (brown) that lines the nasal capsule. The pale area in the front two lamellae represents the folded layers of the olfactory epithelium that are separated internally by the lamina propria. New lamellae are formed in the area of the nasal capsule (not shown). The left box outlines the area shown in Fig. 5a–c. The right box outlines the area reconstructed in Fig. 4.

unify results on components of the sense that have been studied separately in a variety of taxa, allowing us to suggest an outline description of the magnetic sense in vertebrates.

Our results suggest that a magnetite-based magnetic sense makes an important contribution to long-distance orientation by animals. Responses to changes in magnetic intensity have been implicated in the formation of a 'magnetic map'^{1,7,26,27}. The behavioural threshold to magnetic intensity changes necessary to form a useful magnetic map can be no more than a few tens of nanotesla (nT). Thresholds of 10–200 nT have been shown experimentally in some birds²⁶ and honeybees²⁸ and inferred for homing pigeons²⁹ and whales^{30,31}. Such sensitivities can only be achieved by averaging the responses of large numbers of magnetite-based magnetoreceptors³².

Location of candidate magnetoreceptors in the nose of the trout also has implications for experiments on long-distance orientation in species such as homing pigeons. The apparent physical proximity of magnetoreception and olfaction raises the intriguing possibility that olfactory impairment would also produce magnetic impairment and so might disrupt long-distance orientation, as seems to occur in homing pigeons^{33–35}. Attaching magnets close to the nose could be expected to disrupt magnetic but not olfactory contributions to homing. Our work thus brings a new perspective to the study of magnetic orientation and provides experimental support for the intuitively appealing hypothesis that animals use the magnetic sense for navigation. □

Methods

Discrimination training. Our procedure¹⁶ differed from that in ref. 18 only as required for working with trout. Fish were trained every second day. Trials lasted 15 s and were separated by a variable intertrial interval (average length 2 min) during which the tank was darkened. In S+ (reinforced stimulus) trials, the first response after 15 s brought food. In S– (non-reinforced stimulus) trials, a penalty timer started after 15 s and was reset by each subsequent response until either it 'timed-out' or a total of 60 s of penalty time had accumulated. To control for possible generalized effects of the experimental field on behaviour³⁶, for one fish, the altered field became the reinforced stimulus (S+) and the uniform background field became the non-reinforced stimulus (S–). For the other two fish, S+ and S– were the background and altered fields, respectively.

Electrophysiological recording. Fish were prepared using standard procedures. Extracellular recordings (using glass microelectrodes filled with 4 M NaCl solution and a silver wire reference electrode) of the activity of single units were made from the ros V where it crosses the top of the orbit. Standard electrophysiological apparatus was used to amplify, record and analyse the activity of units.

The earth plate on which the preparation and apparatus were mounted cancelled the vertical component leaving only the horizontal component (~25 µT) of the Earth's magnetic field present in the experimental tank. Horizontal coils placed on either side of the head of the fish and aligned parallel to the residual background field could be energized to produce fields aligned either parallel or antiparallel to the background field. Potential mechanical artefacts caused by switching of the current to the coils were minimized by the alignment of the coils relative to the background field and by use of rubber insulation to isolate the preparation from the rest of the apparatus.

Intracellular magnetite. Intracellular magnetite was detected using reflectance-mode CLSM. Magnetotactic bacteria were used to validate the detection procedure (Fig. 3a), with *E. coli* as negative controls. For trout olfactory epithelium, tissue was embedded in JB4 resin for viewing in reflectance mode (Fig. 3b). For TEM, the olfactory epithelium was embedded in Epon resin and thin sections taken through a reflection to locate the crystal shown in Fig. 3c,d (inset) Figure 3d shows the results of energy dispersive analysis of X-ray emissions of the area of the section immediately surrounding the crystal.

Nerve tracing and three-dimensional reconstruction. Juvenile trout (40–60 mm) were anaesthetized in 0.05% MS 222, measured and decapitated just behind the gill cover. After immersion in fixative for 24 h, DiI crystals were applied to the exposed ros V on one side, the head immersed in fresh fixative and placed in an incubator at 37 °C for up to 25 days.

Optical slices in 2-µm steps were taken through four 100-µm gelatin sections using a Leica TCS 4D confocal laser scanning microscope. For three-dimensional reconstruction, the images were cut and pasted using NIH Image into a 512 × 512 format with the 256 × 256 pixel image centrally located, allowing subsequent alignment of laterally offset data sets. Images were combined by selecting fiducial points and realigning all subsequent optical slices within each data set to form a single 149-image three-dimensional data stack (446 µm wide × 685 µm × 298 µm deep). Three-dimensional reconstruction was done using VoxellView software (Vital Images, Fairfield, Iowa). Rotation of the reconstruction enabled finely detailed tracking of the rami of the ros V entering the olfactory lamellae.

Received 29 August 1996; accepted 17 September 1997.

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Acknowledgements. We thank the Whitehall Foundation, Marsden Fund, New Zealand Lotteries Grants Board, Auckland University Research Committee and School of Biological Sciences for support; W. T. M. Grijters, B. M. Davy, I. MacDonald, V. Ward, A. Young and A. Cantell for technical assistance; and M. E. Bitterman, A. R. Bellamy and G. G. Dodson for helpful comments on earlier drafts of the manuscript.

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Formation of elliptical galaxies at moderate redshifts

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Different cosmological models make specific predictions about the number of elliptical galaxies as a function of redshift¹, so observations can in principle be used to discriminate between those models. Traditionally, elliptical galaxies have been thought to have formed in a single, rapid burst of star formation at high redshifts ($z > 5$), and then evolved quietly—with no significant further star formation—since that time^{2,3}. Yet evidence suggests that at least some ellipticals formed for the merger of two spiral galaxies^{4,5}. It remains unclear which process dominates the formation of elliptical galaxies. Here I use the results of deep optical⁶ and near-infrared^{7–10} images to show that there are fewer galaxies with very red colours than predicted by models in which typical ellipticals have completed their star formation by $z \approx 5$, which means that elliptical galaxies must have significant star formation at $z < 5$. This requirement, combined with constraints on bursts of star formation in lower-redshift galaxies^{11,12}, and the observed properties of galaxies in the redshift range $0 < z < 1$ (refs 13–17), suggests either that ellipticals form at moderate redshifts, where a large initial burst of star formation is shrouded by dust, or that they form through the merging of smaller galaxies.

Very deep images at optical and near-infrared wavelengths provide a sensitive test of models of elliptical galaxy evolution. Passive models in which ellipticals form in a single, rapid burst at high redshift predict the existence of a population of high-redshift galaxies with extremely red colours. In particular, galaxies that have formed the bulk of their stars at high redshifts will have extremely red optical and near-infrared colours when observed at ($1 \lesssim z < z_f$), where z_f is the formation redshift. The red colours are the result of the absence of hot, young massive stars combined with the effect of redshift, which causes the observed near-infrared and optical colours to become redder as they probe farther into the rest-frame blue and ultraviolet. This trend towards redder colours at higher redshifts changes only as the redshift of formation is approached, when the hot and blue massive stars have not yet died out.

The predictions of these models are shown in Fig. 1, which traces the colours of galaxies formed in bursts of star formation at various redshifts for representative cosmologies. This diagram demonstrates that galaxies that have completed a large majority of their star formation by $z \approx 5$ reach extremely red colours of $V_{606} - K > 7$. Galaxies with significant star formation at lower redshifts do not have these colours unless reddened greatly by dust. Therefore the number of objects with $V_{606} - K > 7$ gives an upper limit to the co-moving number density of passively evolving elliptical galaxies with $z_f \geq 5$.

Only recently have surveys become deep enough in both the optical and near-infrared bands to test for the presence of these extremely red galaxies at $z > 1$. This advance is due in large part to the optical images of the Hubble Deep Field (HDF)⁶ that allow detections or useful upper limits on objects with the very faint V magnitudes expected of red objects at these redshifts. These optical images have been followed up by several near-infrared surveys that can be used to determine the number of galaxies in the HDF with very red $V_{606} - K$ colours. One of these surveys provides K magnitudes to very faint limits for $\sim 20\%$ of the HDF⁷. Near-infrared

images of the entire HDF to a somewhat brighter limit have also been obtained and made publicly available⁸, and these have been analysed as part of this work. Two ground-based surveys in different fields provide additional constraints on the number of extremely red galaxies^{9,10}. Although these ground-based surveys are not as deep as the HDF surveys, particularly at optical wavelengths, they are valuable because they are in completely independent fields, widely separated on the sky.

The results of these surveys are compared with the predictions of passively evolving models in Fig. 2. The surface density of extremely red galaxies objects is far below the expectations of a model in which all early-type galaxies form in bursts at high redshift. The predictions for the surface density of extremely red galaxies are calculated by using a constant co-moving number density of early-type galaxies set by studies of the K -band luminosity function at low redshift¹⁸, the stellar populations models discussed earlier, and the appropriate cosmology for each plot. This calculation might

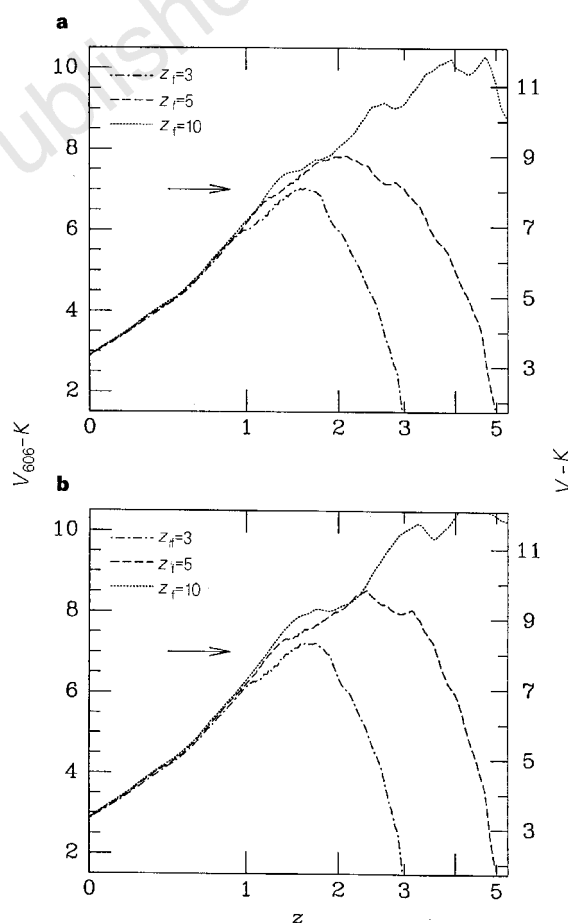
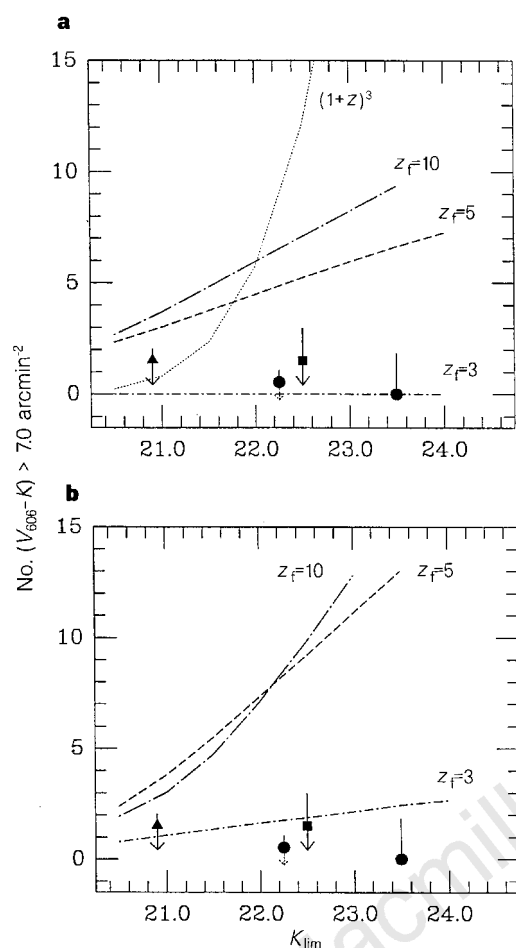


Figure 1 A plot of the observed $V_{606} - K$ colour as a function of redshift for a stellar population formed in a single short burst ($\tau = 0.01$ Gyr): **a**, $\Omega = 1.0$, $h = 0.50$; **b**, $\Omega = 0.2$, $h = 0.65$. Galaxies with higher formation redshifts ($z_f \geq 5$) have extremely red colours that are not reached by galaxies with lower formation redshifts ($z_f \leq 3$). The arrow marks the colour cut used in Fig. 2. Both the closed model plotted in the top panel and the open model in the bottom panel exhibit the same effect. An approximate translation for the HST V_{606} bandpass to the standard V bandpass is given on the right side of the plots. These figures are based on the Bruzual and Charlot models (unpublished data) with solar metallicity and a Miller-Scalo stellar initial mass function (IMF). The results are not sensitive to the IMF within the usual range of parameters (for example, Salpeter versus Miller-Scalo), and a short burst duration is used only to isolate the effect of the formation redshift. For simplicity, absorption by intervening H I has not been accounted for²⁶, which will only make the colours redder at $z \geq 3$.



underestimate the number density of extremely red galaxies expected in passively evolving models because the adopted local luminosity function has a density normalization about a factor of two lower than more global measures at modest redshifts¹⁹. Alternatively, the calculation might be an overestimate in that the luminosity function used includes both elliptical and S0 galaxies, whereas passive models could be said to only apply to ellipticals, which are ~50% of the total.

The strong deficit of galaxies with extremely red colours, seen in widely separated fields and at different flux limits, rules out models in which typical elliptical galaxies are fully assembled and have formed all of their stars at $z \geq 5$. This constraint cannot be escaped simply by breaking ellipticals into many pieces that formed their stars at high redshift. Near-infrared surveys are now deep enough to detect the individual pieces, and thus such models dramatically overproduce red galaxies within the observed range of K magnitudes. An illustrative example with $z_f = 5$, a constant total stellar mass in the early-type population and a co-moving number density of this population that increases as $(1+z)^3$ is plotted in Fig. 2. Models with more modest density evolution follow curves between this model and the $z_f = 5$ passive model with constant number density. Therefore typical elliptical galaxies must have had significant star formation at $z < 5$.

Single-burst models at lower redshifts are strongly constrained by other observations. In particular, the modest ultraviolet luminosities observed for galaxies in the HDF^{11,12}, as well as the failure of searches for strong-emission-line objects²⁰ rule out these models unless the objects are obscured greatly by dust^{21,22}. Dusty models produce high luminosities in the far infrared and submillimetre range, and are close to current observational limits at these wavelengths²².

Alternatively, the simple passive model at high redshift can be

Figure 2 The surface density of extremely red galaxies ($V_{606} - K > 7$) at different limiting K magnitudes compared with the predictions of passive models of elliptical galaxy formation: **a**, $\Omega = 1.0$, $h = 0.50$; **b**, $\Omega = 0.2$, $h = 0.65$. The data points are inconsistent with $z_f \geq 5$ models, including the light dotted line representing a pure density evolution model with $z_f = 5$. The two circles represent surveys in HDF. The deeper of these is the Hogg *et al.* survey with the Keck telescope of $\sim 1 \text{ arcmin}^2$ to a 50% completeness limit of $K \approx 23.5$, in which no objects with colours as red as $V_{606} - K > 7$ are found, and only one is even within one magnitude of this colour⁷. The second HDF data point is based on the publicly available Kitt Peak imaging survey⁸ that covers the full 5 arcmin^2 of the HDF, to a 50% completeness limit of $K \approx 22.25$. Using an updated version of the SExtractor image analysis program²⁷, we find that all sources with $K < 22$ are detected in the HDF optical images and have significantly bluer colours than the passive model predictions. At the fainter limit of $K < 22.5$, there is one good candidate and two marginal candidates for objects with $V_{606} - K > 7.0$. The point plotted accounts for all three of these candidates and is therefore shown as an upper limit. The square and the triangle are from ground-based surveys in other fields by Moustakas *et al.*⁹ and Cowie *et al.*¹⁰, respectively. The former covers $\sim 2 \text{ arcmin}^2$ to a 50% completeness limit of $K \approx 22.5$. Within these limits there are three galaxies that are undetected in V , with approximate lower limits to their $(V - K)$ colour of 7.5, 6.1 and 5.3 (ref. 9). These three represent an upper limit to the number of extremely red objects in this field. The latter survey covers $\sim 6 \text{ arcmin}^2$ to a limiting K magnitude of 20.9. Because the bluer bandpass is not very deep in these data, I adopt $(I - K) > 4.5$ as the best estimate of the equivalent $V_{606} - K > 7$ cut used above. There are approximately nine galaxies with colours this red. This number is more properly considered an upper limit, as there are several objects with red $(I - K)$ but blue optical colours^{9,10}.

altered to incorporate a much longer starburst duration that extends to lower redshift. To make the colours sufficiently blue, this component must form $\sim 5\%$ or more of the stellar mass of ellipticals from z_f to $z = 1$. For example, if 5% of the stellar mass forms in an additional star-formation component that is constant from z_f to $z = 1$ and then shuts off, the resulting $V_{606} - K$ colour is slightly redder than 5 over most of this redshift range. If the mass in the extended component is only a few per cent, then the $V_{606} - K$ colour is greater than 6 and far more red galaxies are expected than observed. Thus, the extended star formation must represent at least 5% of the stellar mass to accommodate the absence of very red galaxies. This modification of the passive model runs into several problems with other observational constraints. First, it increases the evolution to brighter luminosities at higher redshifts expected in passive models, contrary to the results of deep, red-selected galaxy redshift surveys, which find little or no luminosity evolution¹³⁻¹⁵. Moreover, an extended star formation history is unable to account for the elemental abundance ratios in ellipticals¹⁶.

A more natural answer is that most ellipticals form at $z < 5$ through merging and associated starbursts, which are likely to be at least moderately dusty. This result is consistent with a large body of other evidence that indicates that merging has a major role in the formation of ellipticals, ranging from detailed studies of nearby galaxy mergers to the discovery of bimodality in the globular cluster systems of elliptical galaxies¹⁷. The formation of elliptical galaxies in this way is consistent with the predictions of hierarchical clustering models of galaxy formation²³⁻²⁵. □

Received 9 July; accepted 7 October 1997.

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Acknowledgements. I thank colleagues at Berkeley, Cambridge and Durham for stimulating conversations; L. Moustakas for assisting with the use of image analysis software; R. Bouwens for providing code to manipulate stellar populations models; and D. Hogg for providing data in tabular form. This work has been supported by NASA grants.

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Layering of a liquid metal in contact with a hard wall

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When a liquid makes contact with a solid wall, theoretical studies^{1–4} indicate that the atoms or molecules will become layered adjacent to the wall, giving rise to an oscillatory density profile. This expectation has not, however, been directly verified, although an oscillatory force curve is seen for liquids compressed between solid surfaces⁵. Here we present the results of an X-ray scattering study of liquid gallium metal in contact with a (111) diamond surface. We see pronounced layering in the liquid

density profile which decays exponentially with increasing distance from the wall. The layer spacing is about 3.8 Å, which is equal to the repeat distance of (001) planes of upright gallium dimers in solid α -gallium. Thus it appears that the liquid near the wall assumes a solid-like structure similar to the α -phase, which is nucleated on freezing at lower temperatures. This kind of ordering should significantly influence flow, capillary osmosis, lubrication and wetting properties^{5,6}, and is likely to trigger heterogeneous nucleation of the solid.

Wall-induced oscillations in the atomic density are difficult to detect because they occur at a deeply buried interface over a depth interval of typically less than a nanometre. An earlier study of an electrolyte–silver (111) interface showed layering of the water molecules close to the electrode⁷. This ordering phenomenon, however, relates to the presence of a strong electric field at the interface, causing the dipolar water molecules to be preferentially oriented and attracting them to the silver surface. For the free surfaces of liquid Hg and Ga, the in-plane pair distribution function⁸ and the density profile along the surface normal^{9,10} have been determined. In the latter case the strong gradient in the density dependent one-body potential across the liquid–vapour interface, which acts as an effective force on the surface ion cores, is responsible for a layering effect²⁵. The layering reported here is present in a nonpolar elemental liquid lying against a hard wall, in the absence of an external field. This reflects the commonly occurring situation of having a liquid metal contained in a crucible.

Our choice of materials is convenient for several reasons: Ga is liquid at 300 K (supercooled by 8 K) and diamond is essentially

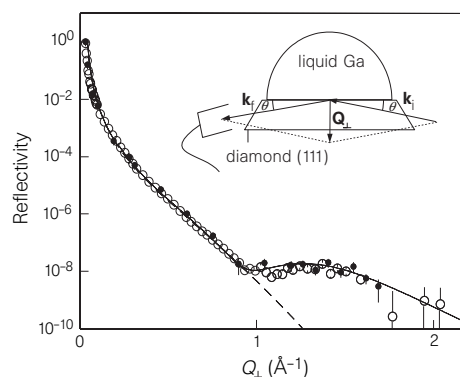


Figure 1 Reflectivity of the Ga/diamond (111) interface as a function of perpendicular momentum transfer $Q_{\perp}$. Measured reflectivities obtained from transverse momentum scans and θ - 2θ scans are represented by filled and open circles, respectively. The solid curve is the best-fit reflectivity curve, obtained by Fourier transform of the electron density profile of Fig. 2; see also equation (1). The dashed curve is a model calculation for a non-oscillatory density profile, see text. The inset shows a schematic of the scattering geometry. The incoming beam is represented by the wavevector $\mathbf{k}_i$, the outgoing beam by $\mathbf{k}_s$. The interface is illuminated from the substrate side with the monochromatic X-ray beam. The top surface of the liquid and the bottom surface of the substrate are shielded from the incoming beam. After scattering, the photons travel through the crystal again on their way to the detector. The momentum transfer $\mathbf{Q}$, the difference between the wavevectors of the incident and scattered photons, is normal to the interface. Note that the index of refraction of Ga is smaller than that of diamond. As the cross section of carbon for Compton scattering is relatively large and a significant volume of bulk liquid is illuminated, there is a high background of diffuse scattering. For the highest momentum transfers, the signal-to-background ratio is a few per cent. At a photon energy of 17 keV, the beam is attenuated by a factor of four along the total pathlength travelled in the diamond. The data were corrected for a 12% variation in the attenuation over the $Q_{\perp}$ range covered. These experiments have only become feasible because third-generation synchrotron sources deliver such brilliant beams of energetic X-rays that sufficient count-rates can be obtained from the small ordering effects in a few atomic layers.

transparent to X-rays at the photon energy used (17 keV), enabling the incident beam to reach the interface from the diamond side (inset of Fig. 1). The diamond (111) substrate, having a high surface free energy, is a prototypical hard wall, which at 300 K is nonreactive towards Ga and is stable against bond breaking¹¹. However, the preparation of an atomically clean interface requires a special procedure involving the controlled deposition, in an ultrahigh vacuum (UHV) environment, of a drop of liquid metal free of surface oxide onto a horizontally positioned clean substrate. The X-ray scattering experiment is to be done *in situ*, so that contaminants from the ambient will not be absorbed by the liquid drop. We have constructed a dedicated UHV set-up which fulfils all of these requirements²⁶. The set-up is fitted with a cylindrical Be-window which is transparent to X-rays and provides access to a large portion of reciprocal space.

The C(111) substrate was a synthetic, type-Ib diamond crystal, of which the surface ($\sim 41 \text{ mm}^2$) was abrasively and manually polished¹². The polished surface had a small misorientation with respect to the (111) plane; its normal tilted by 0.3° towards the [100] azimuthal direction. Subsequent flash-heating in the UHV set-up to $1,000^\circ\text{C}$ resulted in an atomically clean (2×1)-reconstructed surface¹². A millimetre-sized drop of liquid Ga (99.9999% purity) was deposited onto the diamond surface by gently pushing the plunger of a Ga-filled alumina 'syringe' positioned directly above the surface¹³. The first drops from a fresh load usually have an oxide skin. We therefore deposited them in a tantalum drip tray which can be rotated in front of the surface. After three or four drops, the Ga drops were atomically clean, as was checked earlier by Auger electron spectroscopy in a separate apparatus. We then retracted the tray and deposited a clean drop onto the surface. The wetting angle was found to be $116(6)^\circ$ and the contact area 9 mm^2 , as measured *in situ* by X-ray transmission and reflection.

The X-ray scattering measurements were made at the undulator beamline ID10 of the European Synchrotron Radiation Facility in Grenoble, France¹⁴. A wavelength of $0.727 \text{ \AA}$ (energy 17.058 keV) was selected. The scattered intensities were measured using a NaI scintillation detector behind slits defining the momentum acceptance to be $0.02 \text{ \AA}^{-1}$ and $0.07 \text{ \AA}^{-1}$ in the horizontal and vertical directions, respectively. Specular reflectivity measurements were made for perpendicular momentum ($Q_\perp$) transfers in the range $0 < Q_\perp < 2.1 \text{ \AA}^{-1}$. Different values of $Q_\perp$ within this range were

selected by tilting the chamber through an angle θ with respect to the horizontal plane and setting the detector at an angle 2θ . At each $Q_\perp$ value, the reflectivity was determined by integrating the scattered intensity in a transverse momentum scan either in the plane of specular reflection or perpendicular to this plane. Such scans enable us to subtract from the signal the intrinsic background arising from elastic diffuse scattering from the Ga bulk liquid^{9,10}. In addition, symmetric $\theta-2\theta$ scans were made at the specular peak and in the background at either side of the peak. Over the entire $Q_\perp$ range the peaks in the transverse scans were found to have a constant width determined by the instrumental resolution, indicating that we have integrated over the true specular intensity only. It was checked that the three procedures, after usual corrections for illuminated area, Lorentz factor and slit settings¹⁵, yielded the same specular reflectivity values to within 10%.

The measured specular reflectivity, shown in Fig. 1 as a function of $Q_\perp$, shows a broad oscillation with a maximum at $\sim 1.5 \text{ \AA}^{-1}$. This is a signature of layerwise ordering in the liquid with a period of $2\pi/1.5 = 4 \text{ \AA}$. In search for the best fit to the data we have considered a variety of density profiles across the interface. For each model, we calculate the specularly reflected fraction $R(Q_\perp)$ of the incoming flux within the Born approximation using the equation

$$R(Q_\perp) = \left(\frac{4\pi r_e}{Q_\perp} \right)^2 \times \left| \int_{-\infty}^{\infty} [f^C(Q_\perp)\rho^C(z) + f^{\text{Ga}}(Q_\perp)\rho^{\text{Ga}}(z)] \exp(iQ_\perp z) dz \right|^2 \quad (1)$$

where $f^C(Q_\perp)$ and $f^{\text{Ga}}(Q_\perp)$ are the atomic form factors and $\rho^C(z)$ and $\rho^{\text{Ga}}(z)$ the in-plane averaged atomic density distributions for diamond and Ga; r_e is the classical electron radius. In the model, which has in total five adjustable parameters, Ga layers are represented by gaussian functions of increasing width on top of a solid substrate represented by an error function. This yields a damped oscillatory density profile close to the interface and a uniform density far away at either side.

The discrete layer structure of the diamond crystal is not incorporated in the model. Because of the miscut, the normal of the physical interface makes a small angle with the line in reciprocal space that connects the bulk Bragg peaks from the diamond crystal.

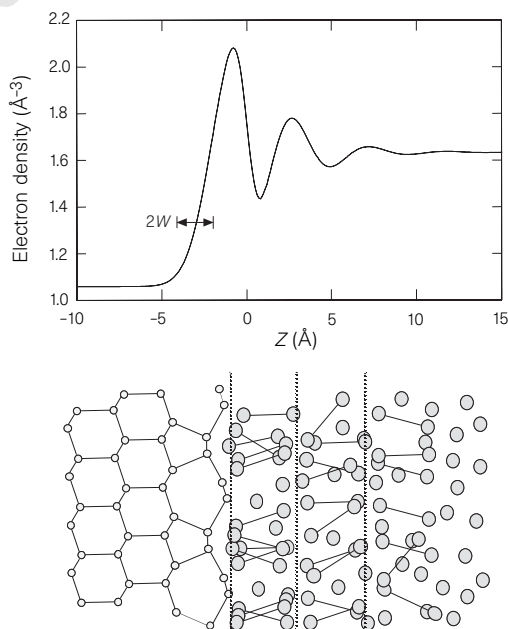


Figure 2 The top panel shows the best-fit model of the oscillatory in-plane averaged electron density profile as a function of the distance z along the interface normal. Far away from the interface, the electron density distributions correspond to the bulk diamond atomic density of 1.77×10^{23} atoms per cm^3 and the bulk liquid Ga atomic density of 5.27×10^{22} atoms per cm^3 . The panel below shows in projection a structural model consistent with the above density profile. Carbon atoms are illustrated by small circles and Ga atoms by larger ones; Ga_2 dimers are represented by two atoms connected by a line. Also drawn are the characteristic five- and seven-membered rings of the π -bonded chain reconstruction of the diamond (111) surface. The model features an accumulation of oriented Ga_2 dimers in increasingly disordered (001) planes of α -Ga. As indicated by the vertical lines, the planes of oriented dimers correspond to successive regions of high electron density (where the dimer ends meet) and of low electron density (at the centre of each dimer).

Therefore, in our $Q_{\perp}$ scans we do not encounter the first Bragg peak at $|Q| = 3 \text{ \AA}^{-1}$ and its tail at smaller Q values¹⁶.

The best fit for the data is obtained for the decaying oscillatory density profile shown in Fig. 2. The oscillation period in the Ga profile equals 3.83 Å. This distance coincides with the distance between two consecutive (001) planes of almost upright oriented Ga₂ dimers in solid α -Ga, which is the stable solid phase of Ga at low temperature and ambient pressure¹⁷. The layering extends into the bulk liquid with a 1/e-decay depth of 4 Å. The areal density of the first layer is larger than that of bulk liquid Ga by 7%. The interface has an overall root mean square (r.m.s.) width W of 1.2 Å (indicated in Fig. 2), which agrees well with the gaussian r.m.s. roughness of 1.4 Å previously measured on the same substrate but without Ga on top²⁷. Also the (2×1) reconstruction seems to be preserved underneath the Ga droplet, as is evident from a striking similarity between non-specular reflectivity curves from both surfaces. The Ga liquid seems to replicate the morphology of the diamond surface. A schematic representation of our model for the layered liquid on top of the (2×1) reconstructed diamond (111) surface is given in Fig. 2.

We have considered other possible structural models for the diamond–Ga interface. For example, specific distributions of atomic step disorder at the interface may give rise to oscillations in the reflectivity¹⁶. However, we measured non-specular reflectivity curves on both Ga-covered and clean surfaces (not shown) but found no such oscillations. Instead, we find for both surfaces identical monotonically decaying Bragg tails for momentum transfers up to $1.5 \text{ \AA}^{-1}$ (midway between two bulk Bragg points). Within this range of momentum transfers the step disorder can be accounted for by a Debye–Waller factor which includes a gaussian roughness of 1.1 Å. To show the effect of step disorder we include in Fig. 1 a specular reflectivity curve calculated for an interface with no density oscillations at the liquid side. Such curves do not describe the measurements. Another model featuring a uniform liquid boundary layer of a different density only gives a reasonable fit to the data if a 7 Å thick layer of at least 20% higher atomic density is assumed, which is clearly unphysical. Density profiles with layer spacings from solid phases of Ga other than α -Ga do not describe the data either. The predominant nearest-neighbour distance of 2.56 Å present in liquid Ga (ref. 18) yields a reflectivity maximum at $Q_{\perp} = 2.5 \text{ \AA}^{-1}$, which is outside the range of measurement. At these high $Q_{\perp}$ values the intrinsic background from the bulk liquid and the crystal becomes too large.

The decay length of the layering amplitude is roughly equal to that of the pair correlation function for bulk Ga (ref. 18). This indicates that short-range order in bulk liquid Ga and layering at the interface are closely related, even though the layering period is different. It supports the notion that the formation of layers is a geometrical consequence of the requirement to form a sharp interface imposed by the diamond wall. The value of 4 Å found by us is smaller than the large value of 5.8 Å found for the decay depth of surface-induced monoatomic layers at the free Ga surface¹⁰.

We believe that layering of the Ga liquid into a structure similar to that in (001) planes of α -Ga is sterically favoured. Liquid Ga is known to contain a non-negligible concentration of Ga₂ dimers in the bulk, which is a manifestation of a certain degree of covalency in the bonding (interatomic distance of 2.44 Å)^{17,19}. The dimers accumulate at the diamond (111)– 2×1 substrate in nearly upright position, thus forming a few solid-like (001) planes of α -Ga. A possible driving mechanism for this type of interfacial segregation and orientational ordering of dimers is an energy gain in the interfacial bonding resulting from the nearly perfect match (<3%) in distance between adjacent π -bonded chains of atoms in the (2×1) reconstructed diamond (111) substrate and the zigzag arrangement of dimers in α -Ga(001) planes. We note, however, that the energy gain is probably small, because the bonding between Ga and diamond is relatively weak, as judged from the large wetting angle of 116°. Also favourable for the accumulation of dimers may

be the fact that this makes the transition across the interface from metallic to covalent bonding less abrupt.

The observed layering of liquid Ga against diamond (111) may have implications for our general understanding of freezing transitions in atomic metals and highlights the possible role of the container wall in triggering the crystallization. Of all crystal faces on α -Ga, the (001) face has the highest atomic density and is the one most stable against surface melting^{20,21}. It is also known that in a supercooled liquid, the barrier for freezing is smallest against the most dense crystal face²². It is therefore tempting to interpret the observed layering as an atomic-scale manifestation of interfacial freezing. However, contrary to the case of surface melting²³, which pre-empts superheating of the crystal, the interfacial freezing observed is not effective in pre-empting supercooling of the liquid. Apparently, an energetic barrier to bulk freezing remains present, making the interfacial freezing a case of incomplete ‘wetting’ of the melt by the crystal. An energetic barrier has been attributed earlier to an entropy loss associated with the layering²⁴. The possible effects of the enhanced ordering at the interface on the barrier to heterogeneous freezing and on the maximum supercooling interval warrant further investigation. Measurements of the temperature dependence of the layering effect may help us to resolve this issue. □

Received 1 April; accepted 13 October 1997.

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Acknowledgements. We thank C. Norris of the University of Leicester for introducing us to his technique of producing clean Ga droplets. We also acknowledge De Beers Diamond Research Laboratories of Johannesburg for the provision of the specimen and M. Rebak of the Schonland Research Center for polishing the crystal. This work is part of the research programme of the Foundation for Fundamental Research on Matter (FOM) and was made possible by financial support from the Netherlands Organisation for Scientific Research (NWO).

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Superconductivity at 10–17 K in compressed sulphur

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Recent high-pressure studies of condensed matter at extreme densities have uncovered various new phenomena in simple molecular and elemental substances¹. One of the most significant pressure-induced changes in materials properties is the transformation of insulators into metals and superconductors. Previous studies of compressed sulphur indicated transitions to metallic phases at 90 GPa (ref. 2) and 162 GPa (ref. 3). Here we demonstrate that at 93 GPa, elemental sulphur transforms not only to a metal, but also to a superconductor with a transition temperature, T_c , of 10.1 K. Using a highly sensitive magnetic susceptibility technique adapted for megabar-pressure diamond anvil cells, we find that T_c increases linearly with pressure up to 157 GPa. This contrasts with the negative dT_c/dP observed⁴ (at much lower pressures) in the heavier superconducting chalcogenides Se and Te. Moreover, at the transformation in sulphur to a higher pressure metallic phase near 160 GPa, T_c increases from 14 to 17 K. These are the highest reported transition temperatures for an elemental solid. As such, these results may provide crucial tests of mechanisms of superconductivity.

At low pressures, sulphur forms an insulating, wide-bandgap molecular solid consisting of rings of S atoms^{5,6}. Controversial for many years, the structural and electronic properties of sulphur under pressure have been clarified during the past decade^{2,3,7}. In particular, diamond-anvil cell experiments have shown that the material undergoes a sequence of phase transitions within the insulating state. Optical reflectivity measurements provided evidence for a transition to a metallic phase at 90 GPa (room

temperature)². Subsequent X-ray diffraction studies^{3,7} indicated that the transformation is accompanied by a crystallographic change to a base-centred orthorhombic (b.c.o.) structure. A second transition was observed by X-ray diffraction at 162 GPa to a rhombohedral phase (identified as the β -Po structure). Superconductivity in proposed high-pressure metallic phases has long been of interest⁷, especially in view of the observation of superconductivity (but at fairly low temperatures) in the heavier chalcogenides Se and Te (refs 4, 9, 10). High-pressure measurements of superconductivity in Se and Te show evidence for maximum critical temperatures of 5.6 and 7.4 K, respectively, which decrease with pressures (<57 GPa)⁴. Recent theoretical calculations predict that sulphur becomes a superconductor with a T_c of 15 K above 550 GPa in the hypothetical b.c.c. phase¹¹. Superconductivity in the lower-pressure metallic phases has not been predicted.

We have used a highly sensitive magnetic-susceptibility technique¹² together with megabar diamond-anvil cell methods to explore the possibility of superconductivity in the material at very high pressures. Recently, we have extended this technique to study samples in the megabar pressure range¹³. The technique is based on the following principles. The magnetic susceptibility of such materials as superconductors depends on the external magnetic field enclosed in the volume of the sample owing to quenching of the superconductivity and suppression of the Meissner effect at the sample's critical magnetic field. In contrast, the susceptibility of the metallic parts of the high-pressure cell (diamagnetic and paramagnetic) is essentially independent of the external field. Thus, the insertion of the high-pressure cell with the sample in an external magnetic field exceeding the critical value changes the part of the signal produced by the sample, while the background remains practically constant. This fact allows the separation of the signal arising from the sample from the background. We apply a low-frequency ($f = 22$ Hz) magnetic field with an amplitude up to several dozen oersted, which causes the destruction of the superconducting state near the superconducting transition. This in turn leads to a change in the magnetic susceptibility of the sample from -1 up to 0 (SI units) twice in a given period, and produces a modulation of the signal amplitude in the receiving coils at a frequency $2f$. The subsequently amplified signal from the lock-in amplifier is then recorded as a function of temperature on the computer. The T_c is then identified as the point where the signal

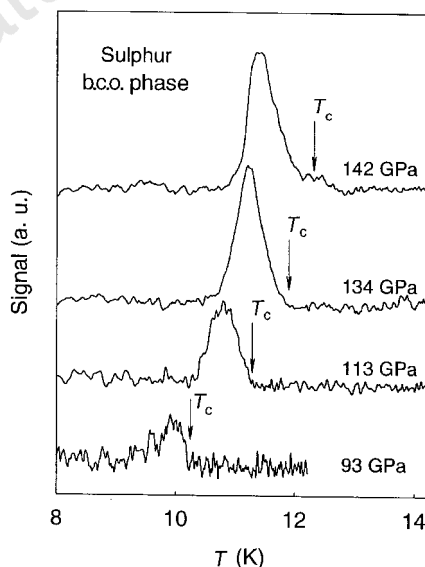


Figure 1 Temperature scans showing the change in magnetic susceptibility (a.u., arbitrary units) near the superconducting transition and identification of T_c (arrow). Uncertainties in T_c are given in Fig. 3.

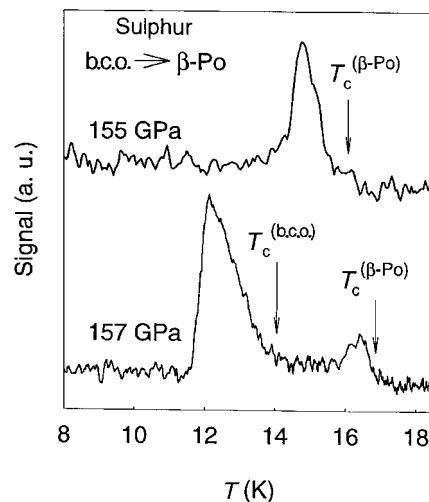


Figure 2 Representative temperature scans at the second phase transition. The measurement at 155 GPa was made after warming to 260 K followed by cooling to 4 K and was obtained several days after the scan at 157 GPa.

goes to zero because of the disappearance of the Meissner effect. We estimate the amplitude of the magnetic field at the sample to be about 50 Oe, and the eddy-current heating should be negligible, as observed in our experiments¹³ on Nb and Ta.

Samples of 99.999% purity sulphur (powder) were loaded in multimegabar diamond anvil cells¹⁴ fabricated from Be–Cu and modified for magnetic measurements down to liquid helium temperatures. Special nonmagnetic stainless steel gaskets were used. Several runs were made with decreasing sample size and diamond culet dimensions to successively higher pressures. In the highest-pressure run, we used bevelled diamonds with 100 μm flats, 500 μm culets, and 10° bevel angle. The sample dimensions were 60–70 μm with a thickness of 8 μm (at ambient pressure). Pressures were determined by the R_1 ruby fluorescence technique (quasi-hydrostatic scale¹⁵) using both Ar⁺ and Ti-sapphire laser excitations *in situ* at low temperatures.

Measurements made as a function of temperature with increasing pressure through the insulating phases revealed no signal (down to 4 K). At 93 GPa, however, a peak characteristic of the transition to the superconducting state^{12,13,16} was observed. Representative temperature scans are shown in Fig. 1. The T_c , identified as the onset of the peak, occurs at 10.1 K. The pressure is close to that reported for the transition identified by optical and X-ray diffraction^{2,3,7}. The temperature and pressure are also close to recently reported evidence for T_c (10 K and 100 GPa) from the loss of resistance¹⁷. Our results indicate that the material transforms directly from an insulator to a superconductor at this pressure. Continuing the measurements to higher pressure within this phase, we find a linear increase in T_c with pressure with a rate of $dT_c/dP = 0.055 \text{ K GPa}^{-1}$. At the highest pressures within this phase $T_c = 14 \text{ K}$ (157 GPa).

Moreover, temperature scans measured at 157 GPa revealed a second well-defined and reproducible peak at 17 K (Fig. 2). This is close to the transition pressure for the second (β -Po-type) metallic phase³. The new peak indicates that the higher-pressure phase has an even higher T_c . The appearance of the two peaks arises for the coexistence of two phases in the presence of a pressure gradient, which is typically observed in such high-pressure experiments¹. The results for T_c are summarized in Fig. 3. The horizontal error bars for the points in the figure correspond to the pressure gradient across the sample measured from ruby chips in the cell. Further increase in

pressure within this phase resulted in a diffuse signal (before diamond failure above 160 GPa). Interestingly, earlier diamond-indentor experiments⁸ using resistance techniques had indicated possible metallization and superconductivity in the material at high pressure. However, these experiments have been controversial because of the uncertainty and inhomogeneity in pressures and (more important) the possibility of shorting of electrical leads. The reported⁸ resistance loss in sulphur near 10 K in such experiments suggests that the same phenomenon may have been measured, although sampling problems precluded unambiguous interpretation.

These results are particularly notable because the metallic phases of sulphur have the highest values of T_c of any elemental solid yet measured. By comparison, for example, the maximum value of T_c for La is 13.8 K at 5.4 GPa (ref. 18), and in the high pressure β -Sn phase of Si at 15 GPa, T_c is reported to reach 8.2 K (ref. 19). Our results now establish that sulphur joins the heavier members of the chalcogenide family, Se and Te, as a superconductor. In contrast to sulphur, T_c for both Se and Te decreases with pressure in their high-pressure orthorhombic phases⁴. The observation of the relatively high T_c in two metallic phases of sulphur, together with the positive dT_c/dP , should provide important tests for theories of superconductivity. Band-structure calculations of the electron–phonon interaction for sulphur predicted a T_c of 15 K, but only above 550 GPa for the hypothetical b.c.c. phase. In the weak-coupling BCS model (see ref. 11), $T_c \propto \Theta_D \exp(-1/N(E_F)V)$, where Θ_D is the Debye temperature, $N(E_F)$ is the density of states at the Fermi energy and V is an effective coupling parameter. The different pressure dependencies observed for sulphur relative to the other chalcogenides suggest that simple scaling of Θ_D (by the square root of the mass) does not hold and that differences in band structure play an important role. It may also be useful to consider the possibility of alternative mechanisms of superconductivity such as electron pairing induced by charge fluctuations, recently examined for elemental hydrogen²⁰. Given the comparative simplicity of elemental sulphur for electronic structure calculations and knowledge of its high-pressure crystal structures, this element should provide important tests of possible new mechanisms. Finally, the strength of the signal and non-invasive character of the magnetic technique opens the prospect for measurements of T_c in such materials at still higher pressures. □

Received 18 July; accepted 30 September 1997.

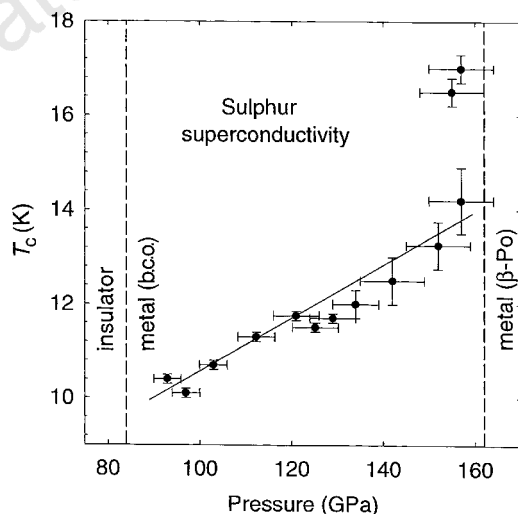


Figure 3 Pressure dependence of T_c in sulphur through the b.c.o. phase and into the β -Po-type phase.

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Acknowledgements. We thank G. Goncharov for extensive help with pressure measurements, and K. Amaya, K. Shimizu and M. Eremets for communicating their results before publication. We also thank P. Jillet and ENS-Lyon for help with manuscript preparation. This research was supported by the NSF.

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Controlling the size, structure and orientation of semiconductor nanocrystals using metastable phase recrystallization

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Materials engineering at the nanometre scale should provide smaller technological devices than are currently available^{1,2}. In particular, research on semiconductor nanostructures with size-dependent optical and electronic properties is motivated by potential applications which include quantum-dot lasers and high-speed nonlinear optical switches^{3,4}. Here we describe an approach for controlling the size, orientation and lattice structure of semiconductor nanocrystals embedded in a transparent matrix. We form nanocrystalline precipitates by implanting ions of the semiconductor into a single-crystal alumina substrate and applying thermal annealing^{5–7}. Control over the microstructure of the nanocrystals is achieved using substrate amorphization and recrystallization. In essence, the substrate microstructure is manipulated using ion beams to induce changes in impurity solubility, crystal symmetry and cation bonding, which exert a profound influence on the microstructure of the embedded precipitates—a concept familiar in metallurgy⁸. This approach can be extended to exercise control over virtually any type of precipitate (such as metals, insulators or magnetic clusters) as well as epitaxial thin films.

Using ion beams to synthesize nanocrystals offers both appealing advantages and materials challenges. Implantation is versatile: almost any ion can be embedded into essentially any substrate with extreme chemical purity (including isotope selection). In addition, as ion implantation can be carried out through masks or with finely focused beams (≤ 10 nm diameter), existing commercial technology can be directly applied with submicrometre pattern resolution. The primary challenges when using implantation to fabricate nanocrystals are controlling the effects of atomic displacement damage caused by the energetic ions and avoiding unwanted reactions between nanocrystals and substrate. Here we show that the atomic displacements caused by the energetic ions can be exploited to provide considerable microstructural control.

The two distinct implantation regimes used to control the nanocrystal structure in this work are shown schematically in Fig. 1. In regime a, lattice damage in the α - Al_2O_3 sapphire substrate is minimized during implantation by using a high substrate temperature (dynamic annealing) or a low ion dose. In this case, the nanocrystals are nucleated within the original α - Al_2O_3 matrix and their microstructure is controlled by the properties of the sapphire

lattice structure. In regime b, the use of cryogenic temperatures or high ion doses causes enough substrate damage to produce amorphization. In this case, the amorphous layer first regrows during thermal annealing as an epitaxial layer of metastable γ - Al_2O_3 (refs. 9, 10). The semiconductor precipitates are subsequently nucleated within this epitaxial, recrystallized γ -layer; consequently, the γ -type nanocrystal microstructure is quite different from the α -type, reflecting the properties of the different host structure. Further annealing transforms the metastable γ -matrix back to the equilibrium α -structure by solid-phase epitaxial regrowth. When this occurs, nanocrystals that are not melted (such as Si) typically retain their γ -type characteristics established during nucleation, whereas nanocrystals of lower melting point (such as Ge) melt and recrystallize as α -type nanocrystals.

The observed structural differences can be understood by considering the related, but distinct, atomic ordering of the α - and γ -alumina matrices. Structurally, α -alumina consists of close-packed oxygen planes arranged in a pseudo-hexagonal ABABAB ... stacking sequence. Each Al atom is located in an octahedral interstice, and is linked to three oxygen atoms in each of the two neighbouring oxygen planes. In contrast, in γ -alumina, the close-packed oxygen planes are arranged in a pseudo-cubic, spinel-like ABCABC ... stacking sequence, with the Al cations occupying both octahedral sites and tetrahedral sites. The recrystallized γ -layers have an epitaxial orientation with respect to the α -substrate. Note that γ - Al_2O_3 is slightly cation-deficient compared with a perfect spinel such as MgAl_2O_4 , and thus should be able to accommodate greater impurity solubility and site-disorder.

Samples were fabricated by implanting semiconductor ions into α - Al_2O_3 (0001) wafers at doses ranging up to $\sim 10^{17}$ ions per cm^2 . Energies for the component ion species of compound semiconductors were chosen to superimpose the concentration profiles. To our knowledge, the only previous studies of high-dose semiconductor ion-implantation in alumina have been reports of precipitation of mullite ($3\text{Al}_2\text{O}_3-2\text{SiO}_2$) or other oxides^{11,12}. As the annealing atmosphere can influence chemical reactions, we carried out post-implantation annealing of the implanted samples in a reducing (96%Ar/4% H_2) atmosphere. Unwanted nanocrystal–substrate chemical reactions were thus avoided by restricting the availability of oxygen.

Quantum confinement effects in semiconductor nanocrystals are determined by the particle dimensions (bandgap shifts of the order of a few electron volts)³, and we have found that nanocrystals formed in an α -matrix are larger and have a larger total volume than

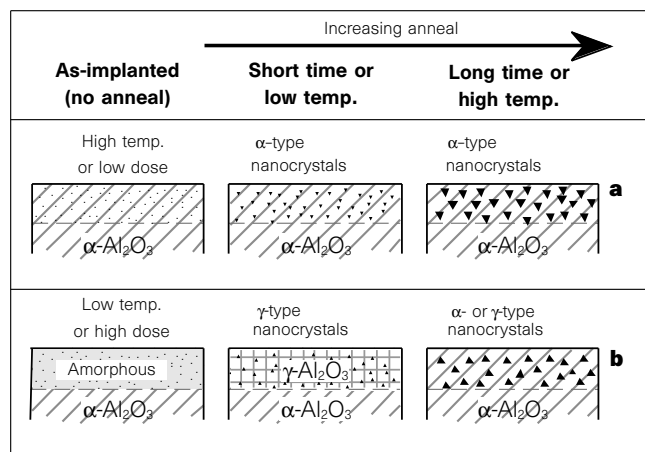


Figure 1 Controlling nanocrystals in alumina. **a**, Substrate remains sapphire during implantation and precipitation; **b**, substrate is amorphized during implantation. Subsequent annealing produces γ -alumina recrystallization, and nanocrystals precipitate in the γ -matrix. Further annealing transforms γ - to α -alumina.

those formed in a γ -matrix under otherwise identical conditions. Figure 2 shows dark-field transmission electron micrographs (TEM) of Ge nanocrystals formed by implanting Ge ions and subsequent annealing at 900 °C. The only difference in the processing conditions was that the substrate temperature during implantation was about 550 °C for the sample in Fig. 2a and liquid N₂ temperature (−195 °C) for the sample in Fig. 2b. The higher-temperature implant contains a high density of relatively large Ge precipitates (up to ~15 nm) embedded in an α -Al₂O₃ matrix, whereas the lower-temperature sample contains significantly smaller Ge precipitates (up to ~7 nm) encapsulated in a γ -Al₂O₃ layer. The magnitudes of the differences depend on the synthesis conditions and species, but $\alpha > \gamma$ size and volume differences are common to all semiconductor species studied. Ratios as large as 100 have been observed.

The greater precipitation in α -alumina can be explained by several possible mechanisms. First, implantation at an elevated temperature can lead to the onset of nucleation (a kinetic 'head-start') even before the subsequent annealing stage. However, the precipitation differences persist even after annealing at much higher temperatures for long times. In addition, we have observed decreased precipitation in a sample in which the γ -layer was pre-formed by Al + O self-ion implantation, ruling out a kinetic 'head-start'. A variety of other kinetic and energetic processes, including damage-enhanced diffusion and interfacial-energy effects, can be involved in nucleation and growth of precipitates. However, our observations are at least consistent with the explanation that enhanced precipitation in α -alumina arises from the almost complete insolubility of most impurities in sapphire. As described above, the increased solubility for impurities in the spinel-like γ -structure can be understood in terms of the availability of additional unoccupied cation sites.

In both matrices, the nanocrystal size distribution depends on the annealing conditions, as is well known in precipitation studies. After an initial diffusion-limited nucleation stage, particle coarsening occurs by Ostwald ripening, a time-dependent kinetic process where the larger particles grow at the expense of the smaller ones¹³. An estimation of the total volume of precipitated material at selected kinetic conditions was obtained by comparing the X-ray integrated intensity with measurements from deposited thin films of known thickness. As expected, the X-ray peaks from precipitates increased in intensity and sharpness with increased annealing. As an example, X-ray scattering from the sample in Fig. 2a accounts for roughly 15% of the implanted Ge ion dose. One consequence of a

single-temperature, Ostwald ripening kinetic process is a relatively broad distribution of particle sizes with a tail towards small particles. It has been suggested that a narrower size distribution could be achieved for technological applications using more complex annealing cycles in which the nucleation and growth processes are activated separately at different temperatures (M. J. Aziz, personal communication).

Both α - and γ -type nanocrystals are found to be crystallographically oriented with respect to the matrix. Whereas the out-of-plane (relative to the surface normal) orientation is the same for both types of nanocrystals, their in-plane (azimuthal) alignments are markedly different. The dominant out-of-plane relation is one in which the densely packed, three-fold (for example {111} for cubic nanocrystals) or six-fold symmetric planes in the semiconductor nanocrystals are parallel to those in the substrate (*c*-planes). Secondary orientations are observed in some cases. A marked difference exists between in-plane crystallographic alignments for nanocrystals in the α - and the γ -substrates. As illustrated in Fig. 2c and d, azimuthal X-ray scans (conventionally designated ϕ scans) reveal the particular in-plane relation, nanocrystal [101] parallel to α -Al₂O₃[11 $\bar{2}$ 0] for cubic nanocrystals precipitated in sapphire. In contrast, nanocrystals precipitated in γ -Al₂O₃ are rotated about the surface normal by $\pm 30^\circ$, corresponding to alignment of the cubic axes of the nanocrystals with the cubic axes of the γ -matrix. Further annealing at a higher temperature transforms the γ -matrix back to the stable α -structure by solid-phase epitaxy. If the γ -type nanocrystals are not melted during this annealing, they remain in the γ -orientation in which they were originally formed. Thus, it is possible for semiconductors such as Si to be reproducibly oriented in either of the two types of directions (α or γ) within an α -sapphire substrate.

The observation that nanocrystals align differently within the two alumina matrices appears surprising at first, as the framework of both substrate structures consists of epitaxially aligned close-packed oxygen planes. Although the alumina frameworks are similar, they have two key differences that influence the nanocrystal alignments. First, the symmetry elements of the two matrices are quite different, and our experimental observations show that semiconductor nanocrystals in alumina orient with their symmetry axes in alignment with those of the matrix. Second, the location of the cation sites within the two matrices are different. The highest-density in-plane rows of cation (Al) atoms in sapphire lie parallel to the close-packed rows of oxygen atoms. In contrast, the closest-packed rows of cation sites within the Mg/Al(111) planes in a perfect spinel are rotated by

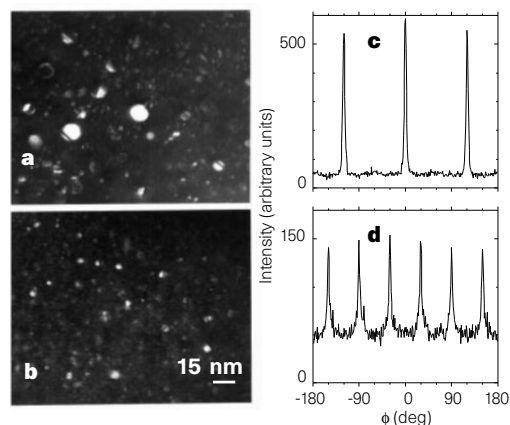


Figure 2 Microstructure of nanocrystals. These nanocrystals were formed by implanting Ge ions (500 keV, $1.5 \times 10^{17} \text{ cm}^{-2}$) into sapphire and annealing for 2 hours at 900 °C. **a**, Dark-field TEM from implant at 550 °C; **b**, dark-field TEM from implant at liquid N₂ temperature; **c**, X-ray ϕ -scan from implant at 550 °C (arbitrary units); **d**, X-ray ϕ -scan from implant at liquid N₂ temperature (arbitrary units).

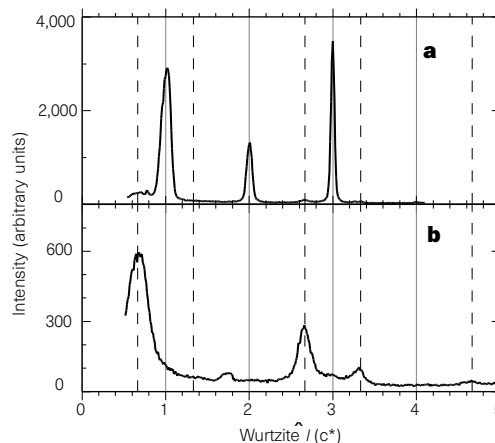


Figure 3 X-ray *I*-scans from CdSe ($4.3 \times 10^{16} \text{ cm}^{-2}$) nanocrystals (arbitrary units). **a**, Implant 600 °C, anneal 900 °C for 1 hour; **b**, implant room temperature, anneal 1,000 °C for 1 hour.

$\pm 30^\circ$ from the oxygen rows. Thus, as atoms at the interfaces of semiconductor nanocrystals are expected to preferentially replace atoms in the cation sites of the matrix, our observations are in accord with simple atomistic models.

These results have important implications for epitaxial semiconductor films grown on the surface of alumina and spinel substrates. Early investigations of silicon-on-sapphire (SOS) films grown on α - Al_2O_3 (0001) surfaces reported inconsistent results and no explanation has emerged¹⁴. To resolve these discrepancies, we note that the reported orientations for silicon films grown on basal-plane sapphire or spinel substrates generally correspond either to the α -type or to the γ -type alignment observed for our embedded precipitates. On the basis of these observations and symmetry considerations, we postulate that the origin of the inconsistencies in previous SOS reports lies in the substrate surface preparation and reconstruction. Surface-damaged sapphire substrates are known to present a spinel-like growth surface¹⁵ and hence may generate γ -oriented epitaxial films, whereas sapphire-terminated surfaces should yield α -oriented epitaxial films. Our results suggest that, as has been accomplished by other approaches¹⁶, it will be possible to pattern different film orientations on a single sapphire substrate.

Compound semiconductor nanocrystals offer an even broader range of possible structural modifications than single-component semiconductors. In addition to the size and orientation effects illustrated above for diamond-cubic, elemental semiconductors, we have found that it is possible to control the lattice structure for compound semiconductor nanocrystals. These results are of importance because many compound semiconductors, such as CdSe and CdS, possess direct or continuously tunable energy gaps with superior electro-optic properties^{3,17,18}. Structurally, bulk CdSe and CdS can be stabilized at room temperature and pressure in either the hexagonal wurtzite (W) structure or the cubic zinc blende structure (ZB). The structural relation between W and ZB can be represented as ABABAB... stacking versus ABCABC... stacking and hence is analogous to the α - γ relation of the oxygen planes in alumina. Previous studies have reported the synthesis of W as well as ZB CdSe nanocrystals¹⁹. However, after a detailed, quantitative analysis, Bawendi *et al.*¹⁸ concluded that nanocrystals previously identified as ZB structure through visual inspection of powder diffraction patterns are actually better fitted by a random stacking sequence of the close-packed planes. In light of this result, there seems to be no previous (unambiguous) identification of CdSe nanocrystals with the ZB structure. Here, we have the experimental advantage of oriented nanocrystals; therefore, we can scan a particular direction in reciprocal space, rather than rely on a powder average.

Figure 3 shows X-ray scans from CdSe nanocrystals in alumina taken along a direction in reciprocal space specifically chosen to probe the stacking sequence. In terms of W coordinates, these scans are along the [0001] direction through the (10 $\bar{1}$ l) reciprocal lattice positions. Indexed in this way, peaks at integral positions indicate the W structure whereas peaks at 1/3 integral positions indicate the ZB structure. The sample in Fig. 3a was implanted at 600 °C and contains CdSe nanocrystals with the W structure, whereas the substrate in Fig. 3b was held at room temperature and contains nanocrystals with the ZB structure. Thus, W nanocrystals are formed in the hexagonal α -alumina host, and ZB nanocrystals are formed in the cubic γ -alumina substrate. We conclude that the nanocrystal lattice structure directly mimics the symmetry of the matrix in which it is formed.

The ability to control the microstructure of semiconductor nanocrystals using ion beams provides opportunities for creating novel devices, and can be generalized to other substrates or other synthesis techniques. Many technologically important materials (ZrO₂, SiO₂, NiAl, YBaCuO, MoSi₂ and so on) have metastable phases and represent prime candidates for consideration as substrates²⁰. Furthermore, we expect that similar effects will prove useful for other types of precipitates, including magnetic materials

and ferroelectrics where anisotropy effects can be exploited using orientational control. Future studies will emphasize techniques for creating nanocrystals with better size uniformity. As self-ordered void arrays are known to form in irradiated sapphire²¹, it is likely that ion implantation techniques can be developed to produce self-organized nanocrystals with uniform sizes. □

Received 8 May; accepted 30 September 1997.

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Acknowledgements. This research was sponsored by Division of Materials Sciences, US Department of Energy, with Lockheed Martin Energy Research Corporation.

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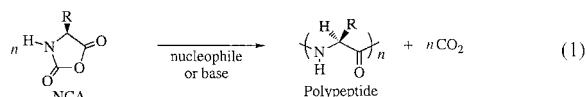
Facile synthesis of block copolypeptides of defined architecture

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Many natural polymeric materials (particularly structural proteins) display a hierarchy of structure over several length scales. Block copolymers are able to self-assemble into ordered nanostructures^{1,2}, but the random-coiled nature of their polymer chains usually suppresses any further levels of organization. The use of components with regular structures, such as rigid-rod polymers, can increase the extent of spatial organization in self-assembling materials³. But the synthesis of such polymeric components typically involves complicated reaction steps that are not suitable for large-scale production. Proteins form hierarchically organized structures in which the fundamental motifs are generally α -helical coils and β -sheets⁴. Attempts to synthesize polypeptides with well-defined amino-acid sequences, which might adopt similar organized structures, have been plagued by unwanted side reactions⁵ that give rise to products with a wide range of molecular weights^{6–10}, hampering the formation of well-defined peptide block copolymers^{11–17}. Here I describe a polymer-

Small peptide sequences, typically less than 100 residues in length, are most conveniently prepared using stepwise solid-phase synthesis. However, the chemical synthesis of high-molecular-weight polypeptides is most directly accomplished by the ring-opening polymerization of α -amino acid-*N*-carboxyanhydride (NCA) monomers (equation (1))^{5,11,12}.



Because there is some chain-length control at low ratios of monomer to initiator, there have been claims that amine-initiated polymerizations can be used to prepare block copolypeptides^{6–10}. These copolymers were often only subjected to limited characterization (for example, amino acid compositional analysis) and, as such, their structures, and the presence of homopolymer contaminants, were not conclusively determined. Copolymers that had been subjected to chromatography showed polymodal molecular-weight distributions containing substantial high- and low-molecular-weight fractions⁶. The compositions of these copolymers were found to be very different from the initial monomer feed composi-

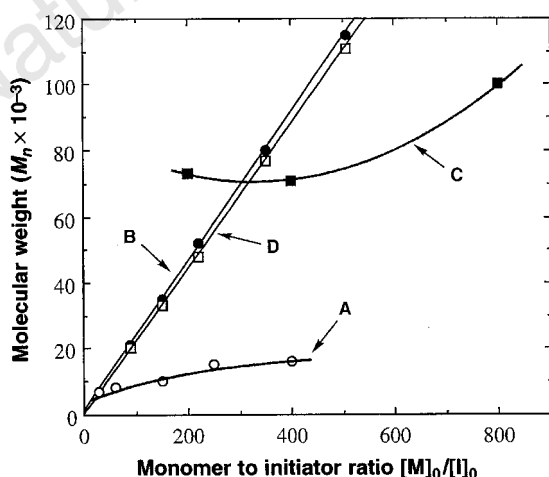
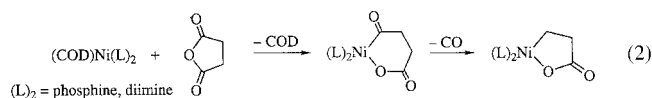


Figure 1 Comparison of the abilities of different initiators to control molecular weight of PBLG as a function of initiator concentration in polymerizations of Glu-NCA: **A**, phenethylamine initiator; **B**, bipyNi(COD) initiator; **C**, sodium *tert*-butoxide initiator; **D**, theoretical molecular weight calculated from $[M]_0/[I]_0$. All polymerizations were run in anhydrous DMF at 25 °C for 1 day in sealed tubes. Molecular weight (M_n) was determined by tandem GPC/light scattering in 0.1M LiBr in DMF at 60 °C.

Successful synthesis of block copolypeptides requires the elimination of side reactions in favour of the chain-growth process (living polymerization), thus allowing multiple monomer additions to each chain^{18,19}. I have approached this problem by using the versatile chemistry of transition metals to mediate the addition of monomers to the active polymer chain-ends¹⁶. The wide range of selective chemical transformations and polymerizations that are catalysed by transition-metal complexes attests to the potential of this approach²⁰. I initially attempted to use metal coordination complexes of conventional amine initiators to control the polymerizations¹⁷. Use of metal-amine complexes for polymerization of γ -benzyl-L-glutamate *N*-carboxyanhydride, Glu-NCA, allowed the preparation of poly(γ -benzyl-L-glutamate), PBLG, with narrow molecular weight distribution ($M_w/M_n = 1.05-1.10$) and some control over molecular weight. However, typical problems inherent in polymerizations initiated by primary amines (slow propagation and chain-transfer reactions) prevented use of these initiators for preparation of block copolypeptides.

$$(\text{COD})\text{Ni}(\text{L})_2 + \text{maleic anhydride} \xrightarrow{-\text{COD}} (\text{L})_2\text{Ni}(\text{maleic anhydride}) \xrightarrow{-\text{CO}} (\text{L})_2\text{Ni}(\text{maleic anhydride}) \quad (2)$$

(L)₂ = phosphine, diimine



Activation and polymerization of NCAs through oxidative ring opening of the anhydride, however, is without precedent. Successful polymerization of L-proline NCA, which lacks a proton bound to

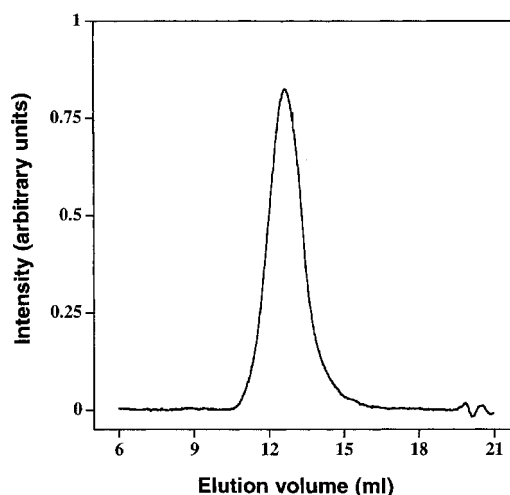


Figure 2 Chromatogram of a PBLG_{0.78}-*b*-PZLL_{0.22} diblock copolymer prepared by sequential addition of Lys-NCA and Glu-NCA to bipyNi(COD) initiator in DMF. The polymerization mixture was injected directly into the GPC, eluted using 0.1 M LiBr in DMF at 60 °C through 10⁵ Å and 10³ Å Phenomenex 5 μ m columns, and detected with a Wyatt DAWN DSP light-scattering detector and Wyatt Optilab DSP.

Table 1 Preparation and analysis of block copolypeptides

First monomer*	Second monomer*	First segment†		Diblock copolymer‡		Yield (%)§
		M_n	M_w/M_n	M_n	M_w/M_n	
52 Lys-NCA	181 Glu-NCA	15,000	1.12	66,000¶	1.21	95
90 Glu-NCA	78 Lys-NCA	28,500#	1.12	52,700**	1.13	93
104 Lys-NCA	40 Leu-NCA	29,500	1.13	34,000	1.20	93
182 Glu-NCA	90 Pro-NCA	57,600#	1.07	86,000#	1.14	92
120 Glu-NCA	40 Leu-NCA	38,000#	1.08	79,000#	1.13	96

Polymerization initiator was bipyNi(COD) in DMF in all cases. Molecular weight (M_n) and polydispersity (M_w/M_n) were determined by tandem GPC/light scattering in 0.1 M LiBr in DMF at 60 °C using dn/dc values (c = concentration) measured in this solvent at λ_0 = 633 nm.

* First and second monomers added stepwise to the initiator; number indicates equivalents of monomer per bipyNi(COD). Leu-NCA = L-leucine-*N*-carboxyanhydride. Pro-NCA = L-proline-*N*-carboxyanhydride.

† Molecular weight and polydispersity after polymerization of the first monomer.

‡ Molecular weight and polydispersity of the complete block copolymer.

§ Total isolated yield of block copolymer.

|| dn/dc = 0.123 mL g⁻¹.

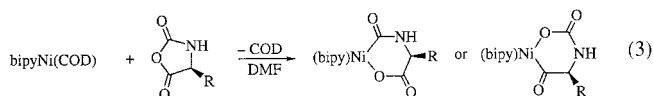
¶ dn/dc = 0.108 mL g⁻¹.

dn/dc = 0.104 mL g⁻¹.

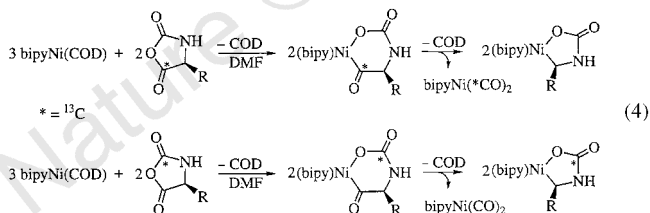
** dn/dc = 0.115 mL g⁻¹.

nitrogen, using bipyNi(COD) supports the hypothesis of oxidative addition across the anhydride bond, rather than reaction at the N–H bond.

As NCAs are unsymmetrical anhydrides, the oxidative addition of NCAs can yield two distinct isomeric products (equation (3)).



I found that the addition of NCAs to nickel was completely regioselective for ring opening across the O–C₅ bond. Reaction of bipyNi(COD) with ¹³C₂-L-leucine NCA and ¹³C₅-L-leucine NCA yielded oxidative addition products and bipyNi(CO)₂ which were examined by ¹³C NMR and Fourier-transform infrared (FTIR) spectroscopy. Detection of bipyNi(¹²CO₂) (FTIR (tetrahydrofuran): CO stretch = 1,978, 1,904 cm⁻¹) from the reaction of ¹³C₂-L-leucine NCA, and bipyNi(¹³CO)₂ (FTIR(THF): CO stretch = 1,934, 1,862 cm⁻¹; ¹³C NMR(DMF-*d*₇): 198 p.p.m. (Ni–CO)) from the reaction of ¹³C₅-L-leucine NCA identified the regiochemistry of the product (equation (4)).



In dimethylformamide (DMF), a good solvent for polypeptides, this addition product was found to be completely active for polymerization of additional NCA monomers.

I analysed the efficiency of the initiator through polymerization experiments with Glu-NCA. The resulting polymer, PBLG, is a α -helical in many solvents, has been extensively studied and is readily characterized²⁴. The number-average molecular weight of PBLG samples formed using bipyNi(COD) in DMF was found to increase linearly as function of the initial monomer-to-initiator ratios, indicating the absence of chain-breaking reactions^{18,19}. Such control over polypeptide molecular weight is a substantial improvement over conventional NCA polymerization systems (Fig. 1). The polymers possessed narrow molecular-weight distributions (M_w/M_n = 1.05–1.15) and were obtained in excellent yields (95–99% isolated). Kinetic analysis also showed that the polymerizations were well behaved. The polymerizations were first order in monomer concentration over four half-lives in DMF (observed rate constant = $2.7(1) \times 10^{-4} \text{ s}^{-1}$ at 298 K; [bipyNi(COD)] = 0.67 mM) showing none of the complexities of traditional NCA polymerizations. The initiating system displays all of the characteristics of a living chain-

growth process for Glu-NCA. Analysis of other NCA monomers (such as ϵ -carbobenzoyloxy-L-lysine *N*-carboxyanhydride, Lys-NCA) also yielded controlled polymerizations, illustrating the general utility of the initiating system for preparation of well-defined block copolypeptides with a variety of architectures.

I synthesized diblock copolymers composed of amino-acid components γ -benzyl-L-glutamate and ϵ -carbobenzoyloxy-L-lysine. The polymers were prepared by addition of Lys-NCA to bipyNi(COD) in DMF to afford living poly(ϵ -carbobenzoyloxy-L-lysine), PZLL, chains with organometallic end-groups capable of further chain growth. Glu-NCA was added to these polymers to yield the PBLG–PZLL block copolypeptides. The evolution of molecular weight through each stage of monomer addition was analysed using gel permeation chromatography (GPC), and data are given in Table 1. Molecular weight was found to increase as expected on growth of each block of copolymer while polydispersity remained low, indicative of successful copolymer formation²⁵. The chromatograms of the block copolypeptides showed single sharp peaks illustrating the narrow distribution of chain lengths (Fig. 2). Copolypeptide compositions were easily adjusted by variation of monomer feed compositions, both being equivalent. Successful preparation of copolypeptides of reverse sequence (PZLL–PBLG) and of triblock structure (such as PBLG_{0.39}–*b*-PZLL_{0.22}–*b*-PBLG_{0.39}; M_n = 256,000, M_w/M_n = 1.15) illustrate the potential for sequence control using the nickel initiator.

Block copolymerizations were not restricted to the highly soluble polypeptides PBLG and PZLL. I have also prepared copolypeptides containing L-leucine and L-proline, both of which form homopolymers which are insoluble in most organic solvents (such as DMF). Data for these copolymerizations are given in Table 1. Because of the solubilizing effect of the PBLG and PZLL blocks, all of the products were soluble in reaction media, indicating the absence of any homopolymer contaminants. The block copolymers containing L-leucine were found to be strongly associating in 0.1 M LiBr in DMF, a good solvent for PBLG and PZLL. Once side-chain protecting groups are removed, the assembly properties of these materials are expected to make them useful as tissue engineering scaffolds, drug carriers and morphology-directing components in biomimetic composite formation. □

Received 27 June; accepted 7 October 1997.

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Interrelated influence of iron, light and cell size on marine phytoplankton growth

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The sub-optimal growth of phytoplankton and the resulting persistence of unutilized plant nutrients (nitrate and phosphate) in the surface waters of certain ocean regions has been a long-standing puzzle^{1,2}. Of these regions, the Southern Ocean seems to play the greatest role in the global carbon cycle^{3,4}, but controversy exists as to the dominant controls on net algal production. Limitation by iron deficiency^{4,5}, light availability^{1,6,7} and grazing by zooplankton² have been proposed. Here we present the results from culture experiments showing that the amount of cellular iron needed to support growth is higher under lower light intensities, owing to a greater requirement for photosynthetic iron-based redox proteins by low-light acclimatized algae. Moreover, algal iron uptake varies with cell surface area, such that the growth of small cells is favoured under iron limitation, as predicted theoretically⁸. Phytoplankton growth can therefore be simultaneously limited by the availability of both iron and light. Such a co-limitation may be experienced by phytoplankton in iron-poor regions in which the surface mixed layer extends below the euphotic zone—as often occurs in the Southern Ocean^{6,7}—or near the bottom of the euphotic zone in more stratified waters. By favouring the growth of smaller cells, iron/light co-limitation should increase grazing by microzooplankton, and thus minimize the loss of fixed carbon and nitrogen from surface waters in settling particles^{9,10}.

We examined the iron uptake and growth dynamics of coastal diatoms (*Thalassiosira pseudonana* and *T. weissflogii*) and dino-flagellates (*Prorocentrum minimum* and *P. micans*) representing a range of cell diameters (3.5 to 30–32 μm) (Fig. 1). Experiments were run at light intensities of 500 and 50 $\mu\text{Einsteins m}^{-2} \text{s}^{-1}$ for *T. pseudonana* and *P. minimum* and at the higher intensity only for the other two species. The cells were grown at 20 °C on a 14:10 h light:dark cycle in EDTA-metal ion buffered media¹¹. We measured the steady-state cellular Fe, Fe uptake rates, chlorophyll *a*, cell size

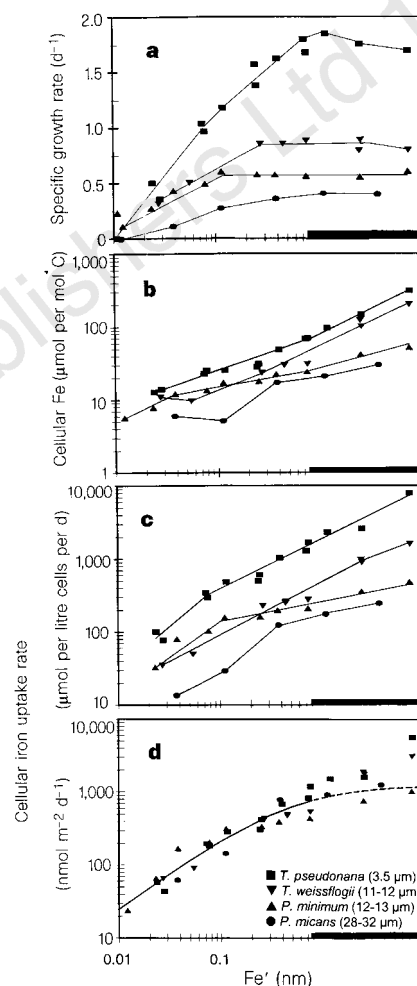


Figure 1 Relationships among specific growth rate, intracellular Fe:C (measured with radiotracers), Fe uptake rate (normalized to cell volume and equivalent spherical surface area), and $[\text{Fe}']$ for coastal eukaryotes of varying mean diameters grown at 500 $\mu\text{Einsteins m}^{-2} \text{s}^{-1}$ (here μE indicates microeinstein). The 36‰ salinity media was enriched with EDTA/trace metal ion buffers and 32 μM NaNO_3 , 2 μM Na_2HPO_4 , 40 μM Na_2SiO_3 , 10 nM Na_2SeO_3 , 0.074 nM vitamin B_{12} , 0.4 nM biotin and 60 nM thiamin. Solid bar on the x-axis represents the region where Fe hydroxides are observed to precipitate¹¹. Horizontal dimensions in all cases are proportional to total Fe. $[\text{Fe}']$ is the mean value over a 14:10 light:dark cycle and equals total dissolved Fe times 0.0025, 0.00166 and 0.00132 at light intensities of 500, 160 and 50 $\mu\text{Einsteins m}^{-2} \text{s}^{-1}$. The solid curve in **d** gives the modelled saturation curve as fitted to equation (1) by nonlinear regression ($R^2 = 0.90$) using data for $[\text{Fe}'] \leq 0.75$ nM. Results presented here are mostly new, but also include some published¹¹ high-light data for *T. pseudonana*, *T. weissflogii* and *P. minimum*. The data include three separate experiments with *T. pseudonana*, and two each with *T. weissflogii* and *P. minimum*. A single *T. pseudonana* experiment with four Fe levels run in triplicate yielded mean standard deviations of ± 3.2 , 3.9, 8.5, 8.4 and 10% for specific growth rate, Fe:C, Fe per litre cell volume, and C- and volume-normalized uptake rates, respectively. Experimental procedures and analyses are as described previously¹¹.

and specific growth rates over a range of concentration of dissolved inorganic Fe species ($[\text{Fe}']$)¹¹, the primary parameter that controls Fe uptake in eukaryotic marine algae^{8,12,13}. Studies of growth rate only were made at low light with *P. micans* and at three light intensities with the coastal cyanobacterium *Synechococcus bacillaris*.

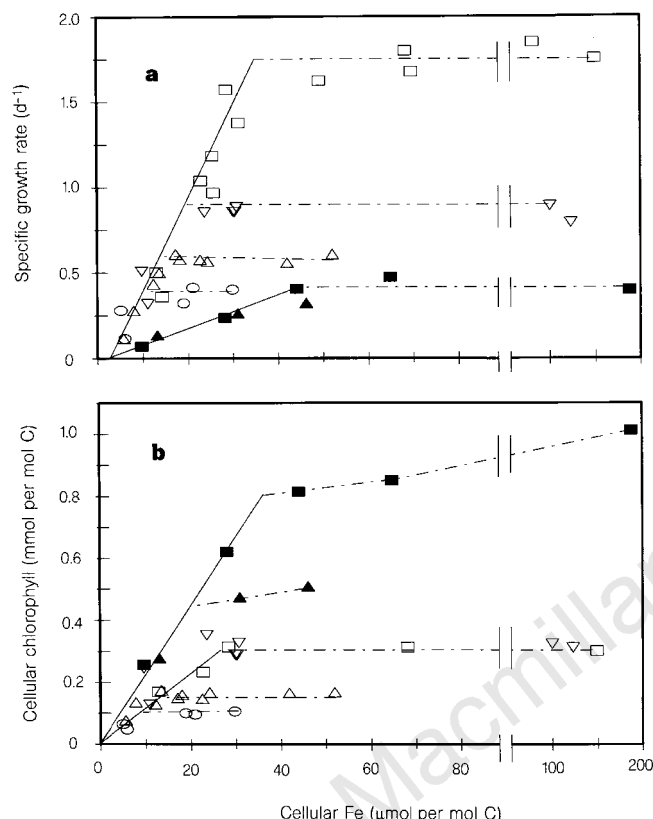


Figure 2 Specific growth rate (a) and Chl a : C (b) as functions of intracellular Fe : C at high (500 $\mu\text{E m}^{-2} \text{s}^{-1}$; open symbols) and low light (50, closed symbols). Light was controlled by neutral density screens. Cellular parameters were measured as described previously¹¹. Symbol shapes are as defined in Fig. 1d.

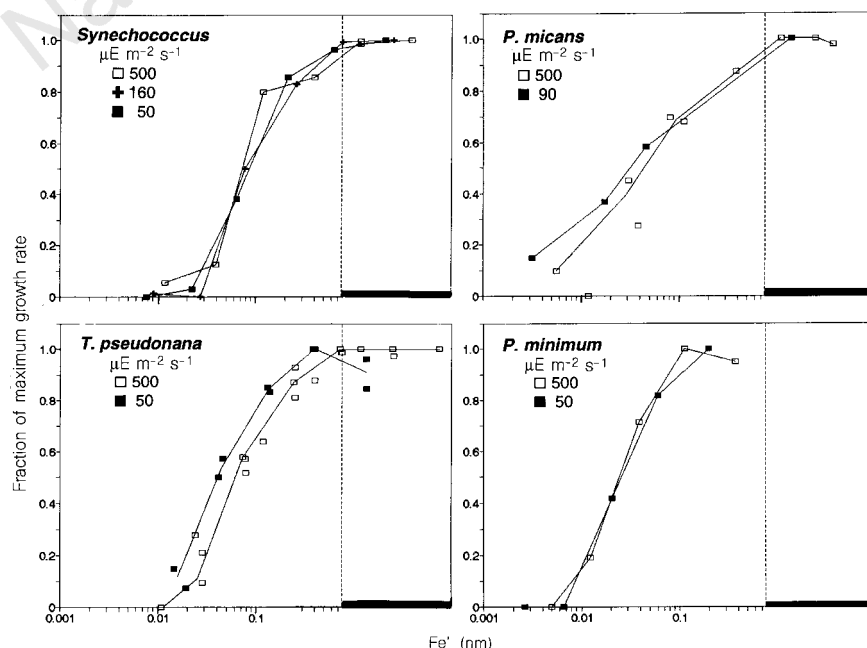


Figure 3 Plot of μ/μ_{max} against $[\text{Fe}']$ at growth-saturating and growth-limiting light intensities. The vertical dotted line marks the solubility limit for $[\text{Fe}']$.

At high light, Fe limitation of growth rate in all four eukaryotic species occurred at or below the measured solubility limit for $[\text{Fe}']$ with respect to Fe hydroxide precipitation¹¹ (Fig. 1a), above which $[\text{Fe}']$ should be constant. Growth rate was inversely related to cell size under both Fe-sufficient and Fe-limiting conditions.

Cellular Fe normalized to carbon (Fig. 1b) and to cell volume (data not shown) increased with Fe concentration and decreased with cell size. The steady-state uptake rate for Fe, calculated by multiplying cell Fe by specific growth rate, showed an even weaker positive dependence on external Fe and inverse dependence on cell size due to the decrease in growth rate with increasing cell size and with decreasing $[\text{Fe}']$ (Fig. 1c). But when normalized to cell surface area, uptake rate (V_{SA}) in all four species fitted a single saturation equation for nutrient uptake (Fig. 1d):

$$V_{\text{SA}} = V_{\text{max}}[\text{Fe}']/([\text{Fe}'] + K_s) \quad (1)$$

where $V_{\text{max}} = 1,276 \text{ nmol m}^{-2} \text{d}^{-1}$ and $K_s = 0.51 \text{ nM}$. Thus, the larger cells had low volume- or C-normalized uptake rates because of low surface-to-volume ratios. The similarity in surface-normalized uptake rates is predicted from previous findings which show that Fe uptake in coastal and oceanic species occurs at theoretically limiting rates determined by the kinetics of Fe transfer to surface uptake sites and available space on the cell's membrane^{8,11}.

Iron uptake by diatoms continues to increase with total Fe in the region of hydroxide precipitation (Fig. 1d), implying that Fe in the hydroxides is available for uptake¹¹. But the availability of Fe hydroxides is much less than that of Fe' because the ratio of hydroxides to $[\text{Fe}']$ where the additional uptake occurs is $\sim 10,000$.

Under Fe-limitation, specific growth rate (μ) was linearly related to cell Fe : C ratio for all four species (Fig. 2a), consistent with the role of Fe as a catalyst in biosynthesis¹⁴. Linear regression of the high-light data gave the relationship

$$\mu = B([\text{Fe} : \text{C}] - m) \quad (R^2 = 0.91) \quad (2)$$

where m (3 mmol mol⁻¹) is the Fe : C ratio needed for cell maintenance at zero growth rate. The slope, $B = 49,360 \text{ mol C (mol Fe)}^{-1} \text{d}^{-1}$, defines the iron use efficiency (IUE) for a photoperiod of 14 h d⁻¹, which translates to 84,600 ($49,360 \times 24/14$) for continuous light. The latter value compares favourably with the IUE (72,000 mol C (mol Fe)⁻¹ d⁻¹) computed from cell models of the Fe needed to support photosynthesis, respiration and nitrate assimilation,

assuming 1:1:1:1 stoichiometry for photosystem II:cytochrome *b₆/f* complex:cytochrome *c*:photosystem I and that flavodoxin replaces ferredoxin¹⁴. At high Fe:C, growth rates reached maximum values, which were inversely related to cell size (Fig. 2a).

At one tenth of the previous light intensity, the Fe-limited growth rate of *T. pseudonana* and *P. minimum* also fitted equation (2), but the slope of the relationship between μ and [Fe:C] was reduced fivefold to 9,700 mol C (mol Fe⁻¹) d⁻¹ (Fig. 2a). Maximum growth rates were also reduced, but by lower amounts (1.9- to 3.8-fold). The higher Fe:C needed to support growth at low light occurs because of reduced light capture and electron flow per photosynthetic unit (PSU). Cells acclimatize to low light largely by increasing the number of PSUs^{15,16}, which include light harvesting pigments, photo-reaction centres and electron transfer molecules. PSUs contain many cytochromes and Fe/S proteins, and account for much of the cell's ion content^{14,15}. Thus, a low-light adaptive increase in PSUs involves a large increase in cell Fe.

The chlorophyll *a*:C ratio of Fe-limited cells was proportional to Fe:C. The Chl:Fe ratio determined from the slope of the plots of cell Chl *a* against Fe (Fig. 2b) was higher at low light (21.3 mol Chl (mol Fe)⁻¹) than at high light (11.5) owing to photoacclimatization. From IUE models^{14,15}, 50% of the cell's Fe should occur within PSUs at high light and 90% at low light. By dividing cell Chl *a*:Fe by the fraction of Fe in PSUs, and multiplying this value by 24 Fe atoms per PSU¹⁴, we calculate that there are 550 Chl *a* molecules per PSU at high light and 570 at low light, similar to values (500–1,300) observed in coastal eukaryotic species at similar light intensities¹⁶. Our results indicate an approximate stoichiometry for Fe and Chl within PSUs as predicted by Raven¹⁵. The results imply that the well-known decrease in Chl under Fe-limitation results from a decrease in PSUs due to insufficient Fe for synthesis of needed cytochromes and Fe/S proteins.

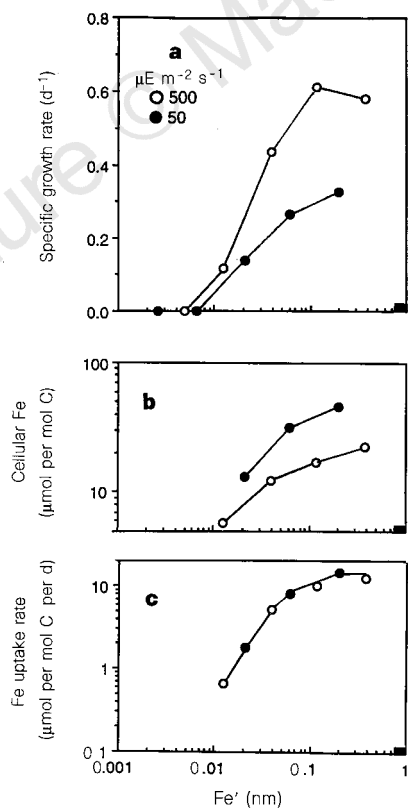


Figure 4 Relationships among specific growth rate, intracellular Fe:C, Fe uptake rate, and $[\text{Fe}']$ for *P. minimum* at growth-saturating and limiting light (500 and 50 $\mu\text{E m}^{-2} \text{s}^{-1}$).

As cells need higher Fe:C for growth under low light, we had expected low-light acclimatized cells to require higher external Fe levels to achieve maximum growth rate (μ_{max}). Despite this expectation, plots of μ/μ_{max} against $[\text{Fe}']$ for the four species tested show similar results irrespective of light intensity (Fig. 3). The reasons for this surprising result can be seen from relationships among specific growth rate, cell Fe:C and C-normalized Fe uptake rate for *P. minimum* (Fig. 4). As light is decreased, μ decreases because of light limitation (Fig. 4a). Because the cells are taking up Fe at the maximum rates permitted by physics and chemistry (see above), the uptake rate at low light for a given $[\text{Fe}']$ cannot exceed that at high light, and indeed relationships between uptake rate and $[\text{Fe}']$ are independent of light intensity (Fig. 4c). The decrease in growth rate with light limitation causes cell Fe:C to increase (Fig. 4b) because of the inverse relationship between Fe:C and μ at constant steady-state uptake rate, V_{ss}

$$\text{Cell Fe:C} = V_{\text{ss}}/\mu \quad (3)$$

This increase allows the cells to synthesize additional PSUs needed for low light acclimatization.

Unlike other potentially growth-limiting nutrients (N, P, Si and Zn) which are highly soluble and can reach high concentrations, levels of biologically available Fe' in sea water are limited by the solubility of Fe hydroxide. Once these hydroxides form, additional Fe inputs will not increase $[\text{Fe}']$, only Fe hydroxides, which are much less available than soluble Fe' (ref. 13) and also will aggregate and settle out from sea water¹⁷. Although most of the dissolved Fe is chelated by organic ligands^{18–20}, the chelated Fe should not be directly available for algal uptake^{8,12} and will not affect the solubility limit on $[\text{Fe}']$. We argue that the low solubility of Fe' places a limit on the amount of metabolic Fe a cell can accumulate for a given size and, thus, on the maximum growth rate it can achieve under various light conditions. Such a limit would be most important in coastal and estuarine systems which receive high Fe inputs from continental sources¹⁷. This idea is supported by our finding that the minimum $[\text{Fe}']$ needed to achieve maximum growth rate just equals the $[\text{Fe}']$ solubility limit in three out of our five coastal species under both saturating and growth-limiting light. In no case does it exceed this limit. We argue that this occurs because over the past ~2 billion years, the maximum $[\text{Fe}']$, and, thus, the maximum growth rate that cells could achieve, were fixed by this limit.

Our results indicate that Fe and light limitation and cell size are all integrally linked. In the open ocean, Fe levels are much lower than in coastal waters and $[\text{Fe}']$ should generally be below the solubility limit^{19–22}. Iron limits algal growth in at least some oceanic systems^{5,23,24} and cells are more likely to be Fe-limited under low irradiance. Adaptation to low light at the bottom of the euphotic zone in stratified regions will lead to high cell Fe:C ratios and, therefore, high removal of Fe relative to other nutrients (C, N and P). This higher removal should drive these systems towards greater Fe limitation, and may largely explain the observed depletion of Fe within deep Chl maxima^{21,22}. The combined Fe/light limitation will favour the growth of small cells, consistent with the observed dominance of small prokaryotes and the paucity of larger eukaryotes in deep Chl maxima^{25,26}.

Martin *et al.*^{4,5} have argued that the limited draw-down of nitrate and CO_2 in the Southern Ocean results from Fe limitation of algal growth, whereas Nelson, Mitchell and others^{6,7} argue that it results primarily from light limitation due to deep mixed layers and low overall solar irradiance. We argue that they are both correct, and that the phytoplankton are simultaneously limited by Fe and light. Furthermore, by favouring the growth of small cells, Fe/light co-limitation should increase grazing rates by microzooplankton and reduce settling rates of algal cells and faecal pellets, and thereby increase nutrient cycling and decrease export of carbon and nutrients from surface waters^{9,10}. □

Received 15 April; accepted 3 October 1997.

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Acknowledgements. This paper was supported by grants from the Office of Naval Research.

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Anisotropic upper-mantle stratigraphy and architecture of the Slave craton

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The Earth's physical properties show a dominantly radial structure which is the result of compositional differentiation, isochemical phase changes¹ and rheological layering². Rheological layering is perhaps the most difficult to investigate using conventional seismological techniques because the seismic manifestation of this property, elastic anisotropy, may closely mimic the effects of isotropic heterogeneity³. Nonetheless, an improved characterization of Earth's rheological structure promises important insights into such processes as plate dynamics and continental

evolution. Here, I present a methodology for effectively characterizing sharp transitions in anisotropic, upper-mantle structure using the coda of teleseismic P-waves. Application to seismograms from the Slave craton reveals a well-developed stratigraphy, at least in part anisotropic, with major boundaries occurring at nominal depths of 75, 135 and 195 km. The geometry and sharpness of these discontinuities suggest a structural origin, perhaps involving shallow subduction.

The Slave province is a small (2.1×10^5 km²) Archaean craton located in Canada's Northwest Territories (Fig. 1), hosting the oldest dated rocks on Earth⁴. These rocks together with recent diamond discoveries in the region⁵ have prompted considerable research interest in the Slave province with the objective of better understanding craton structure and evolution. A remote means of accessing the subcrustal, cratonic lithosphere involves the analysis of seismic energy arriving in the coda of teleseismic P. I have compiled a set of 907 broadband three-component seismograms recorded at five stations (YKW1–4, and RSNT) near Yellowknife, Northwest Territories, representing a total of 427 earthquakes. These events are well distributed in azimuth and epicentral distance (Fig. 1). Each seismogram contains the combined effects of Earth structure and earthquake source; the latter must be removed for the present purpose. Seismograms are source-normalized using an approach similar to conventional receiver function analysis⁶, but incorporating a number of important modifications to improve resolution of subtle features in the seismic wavefield. These include a more effective P, S decomposition through inversion of the free surface transfer-matrix⁷, and simultaneous deconvolution of multiple seismograms to isolate structural effects⁸.

The product of this signal processing is essentially the S-wave contribution at early times to the Earth's impulse response. One may choose to associate this empirical Green's function with either one-dimensional or three-dimensional structure depending on the selection of seismograms used in simultaneous deconvolution. An effective means of presenting and analysing the impulse response is in colour-coded images of amplitude as a functions of time and epicentral distance, as has been used, for example, to characterize the Earth's long-period response⁹. The radial component impulse response for Yellowknife is shown in Fig. 2a from 5 s before to 30 s after arrival of direct P over the epicentral distance range from 30° to 100°. This image comprises a 514-seismogram subset of the

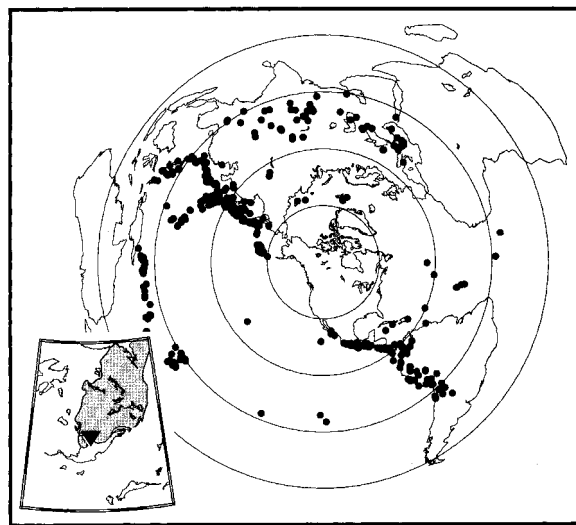


Figure 1 Azimuthal projection of the distribution of events (small black circles) recorded at Yellowknife and used in this study. Inset shows location of Yellowknife stations (triangle) relative to outline of Slave craton in grey. Circles represent 30° increments in distance from Yellowknife.

events in Fig. 1 that occurred between 275° and 360° back azimuth, and has been divided into 0.7° bins in distance. Individual impulse responses for each bin have been filtered between 0.5 and 0.02 Hz; no spatial filtering or averaging has been applied.

A number of coherent features are evident in the image. The direct Ps conversion from the Mohorovičić discontinuity (Moho), P_{MS} is revealed as a positive polarity arrival at ~ 4.7 s. It is followed roughly 10 and 15 s later by P_{PMs} and P_{SMs} , two free surface reverberations from the Moho which are positive (red) and negative polarity (blue), respectively. The two reverberatory phases effectively mask the time interval over which possible direct Ps conversions from the lithospheric upper mantle to depths of 200–250 km might be expected to occur, and thus represent a considerable impediment to the use of P-coda seismograms to image the subcrustal lithosphere, especially at longer periods¹⁰. However, an additional phase with dipolar character is clearly evident between P_{MS} and P_{PMs} at about 7.75 s (9.0 s for the second pulse) in Fig. 2a (labelled P_{HS} in Fig. 2b). An important attribute of this phase is that it possesses negative differential slowness with respect to direct P (travel times decrease by roughly 0.8 s between 30° and 100°). In a laterally homogeneous Earth this is diagnostic of direct Ps conversions; free surface reverberations, in contrast, possess positive differential slowness. The dipolar nature of P_{HS} is most easily explained by way of two P-to-S conversions originating at the boundaries of an apparently high seismic velocity layer (collectively termed H), located between about 75 and 85 km depth. A recent Lithoprobe reflection survey¹¹ has imaged a mantle reflector at similar depth extending laterally some 100 km beneath the southwest Slave craton, including the near vicinity of Yellowknife, thus corroborating the geometrical provenance of the feature.

Further insight into the nature of H can be garnered from an examination of the transverse component impulse response plotted in Fig. 2c. Assuming a laterally homogeneous, isotropic Earth, one would not expect coherent signals to appear on the transverse component. Surprisingly, therefore, several well-defined signals can be traced across the image, all with negative differential slowness, lending strong support to an interpretation as direct Ps conversions. P_{HS} with waveform and amplitude similar to that on the radial component is most prominent, followed by a second reverberatory arrival centred at about 14.5 s (135 km depth, P_{135S}), and a third, single pulse P_{LS} at 21.25 s (195 km depth, where L refers to the 'Lehmann' discontinuity¹²). The appearance of energy on the transverse component attests to the presence of lateral heterogeneity and/or anisotropy. Interpretation of the Lithoprobe reflection line, which runs roughly perpendicular to strike of surface geology, reveals a varying dip on H of up to 6° (ref. 11). Depth-migrated transverse transmissivity profiles as functions of back azimuth plotted in Fig. 3 provide further insights into lateral variations in structure. Here, the signature of lateral heterogeneity is readily apparent; for example, L increases in depth from 150 km at 150° back azimuth near the southern craton margin to >220 km in the more central portions to the north and northeast. The layer sequence at 135 km inferred from P_{135S} also evolves in a complicated manner with evidence for topography and polarity reversal.

Lateral heterogeneity is not the complete picture, however. Closer examination of P_{HS} indicates that it undergoes an obvious polarity reversal at $\sim 270^\circ$ back azimuth and apparently a second one near $\sim 180^\circ$. This suggests a 180° periodicity in polarity that cannot be explained by a dipping layer alone (which would show 360° periodicity¹³) but implies the influence of anisotropy. Confirmation

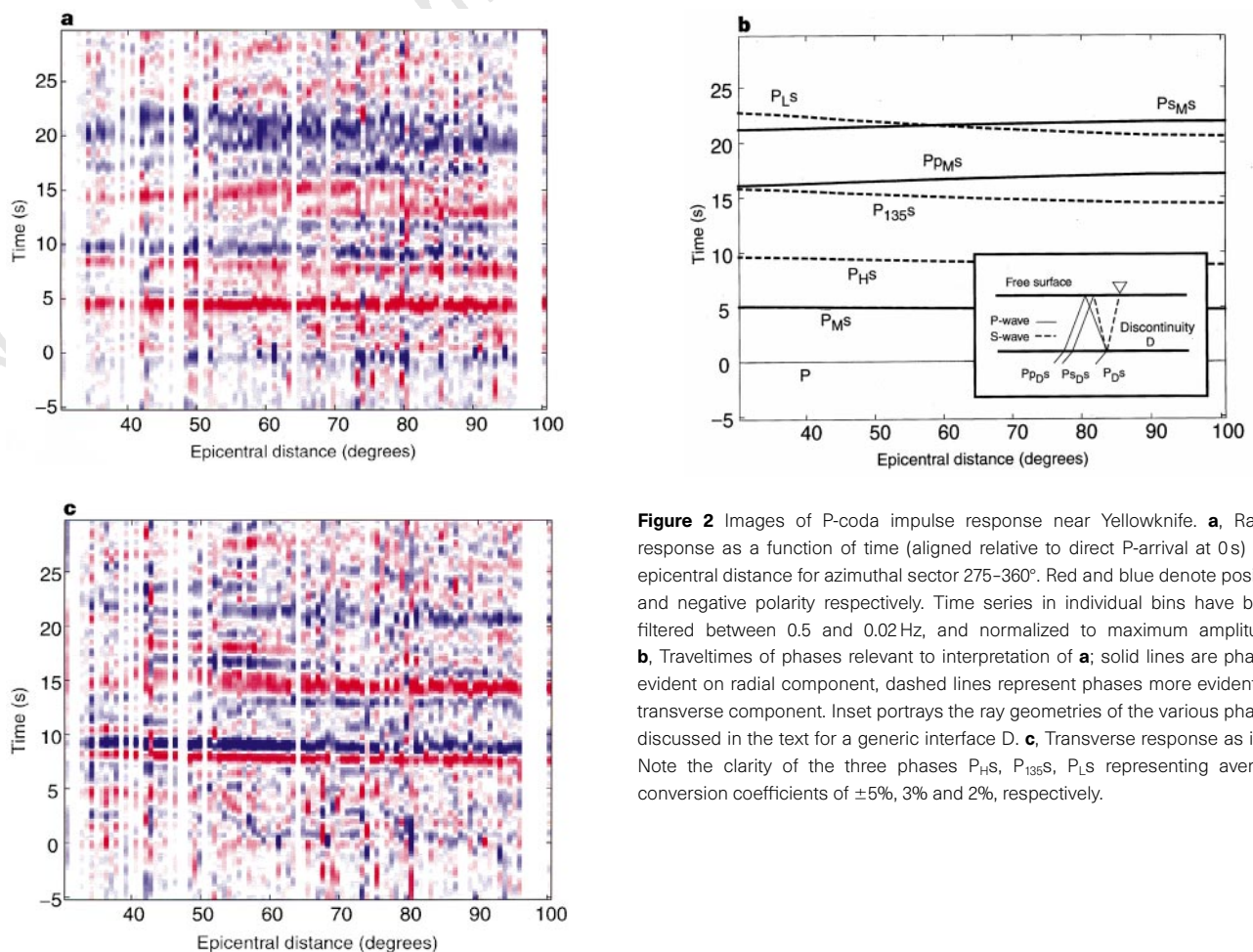


Figure 2 Images of P-coda impulse response near Yellowknife. **a**, Radial response as a function of time (aligned relative to direct P-arrival at 0s) and epicentral distance for azimuthal sector $275\text{--}360^\circ$. Red and blue denote positive and negative polarity respectively. Time series in individual bins have been filtered between 0.5 and 0.02 Hz, and normalized to maximum amplitude. **b**, Travel times of phases relevant to interpretation of **a**; solid lines are phases evident on radial component, dashed lines represent phases more evident on transverse component. Inset portrays the ray geometries of the various phases discussed in the text for a generic interface D. **c**, Transverse response as in **a**. Note the clarity of the three phases P_{HS} , P_{135S} , P_{LS} representing average conversion coefficients of $\pm 5\%$, 3% and 2% , respectively.

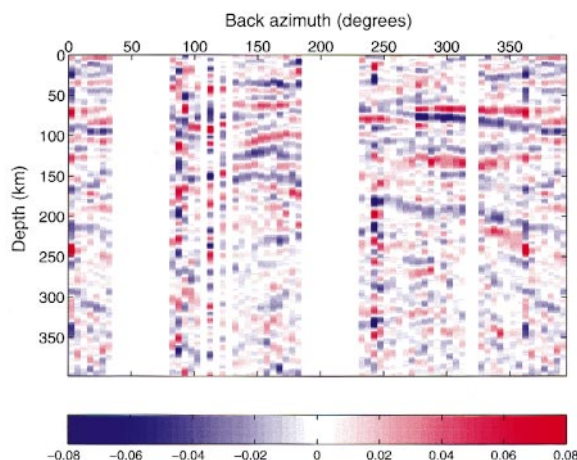


Figure 3 Transverse $P \rightarrow S$ transmissivity as a function of depth and back azimuth. All seismograms within 5° bins in back azimuth are simultaneously deconvolved and migrated to depth using a one-dimensional model of the Earth. Amplitudes are normalized to that of the incident P -wave. Note that deeper discontinuities are sampled at greater radial distances from Yellowknife than shallow ones and so the profile can be viewed as a representative slice of Earth structure along a conical surface extending vertically downwards with focus at Yellowknife (for a discontinuity at 200 km depth, radial distance is of the order of 60 km).

is provided by the observation that H also shows a well-defined polarity reversal(s) on the radial component (Fig. 4), $\sim 45^\circ$ out of phase with those on the transverse component as a function of back azimuth. This is not observed for isotropic, planar, dipping layers¹³ but requires that H represent a layer with some configuration of anisotropic interfaces across which the average shear velocity is comparable. The absence of significant P_{MS} on the transverse component indicates that the mantle immediately underlying the crust is not strongly anisotropic. It is more difficult to ascertain whether strong anisotropy exists in the mantle immediately below H ; however, if strong anisotropy does exist, its orientation must deviate significantly from that within H . In any case, we are led to the conclusion that H represents a layer of depth-localized anisotropy. Reflectivity modelling, assuming a layer of orthorhombic anisotropy with horizontal mirror plane embedded within an isotropic mantle, indicates that shear velocity anisotropy (to which the P_S conversions are most sensitive) within the layer is of the order $\pm 5\%$ (see Fig. 4). Furthermore, directions of polarity cross-over on the transverse component ($270^\circ \pm 90^\circ$) will correspond to the principal axes of anisotropy within H . Note thus that the principle axes are highly oblique to the SKS fast polarization direction of $\sim 50^\circ$ observed at station RSNT¹⁴.

The depth of H associates it with the 'Hales' discontinuity¹⁵ which has been identified on long-range refraction profiles over North America^{15–17} and Australia¹⁸, and from long-period ScS reverberations in the western Pacific¹⁹. The consensus that has emerged is that the discontinuity is best ascribed to the phase transition from spinel to garnet in lherzolitic peridotite, although a perceived difficulty has been the low theoretical velocity contrast. This interpretation of H can be reconciled with the observation of a layer made here, if appeal is made to transformational plasticity²⁰. Phase changes in polyphase peridotite may serve to weaken the mantle over finite transitional intervals allowing for the development of highly strained and anisotropic layers. In this context H could represent a dynamic and evolving component of the craton. An alternative mechanism capable of producing localized strain-induced anisotropy involves the temperature-controlled transition between diffusion and dislocation creep². The implications of this mechanism may be

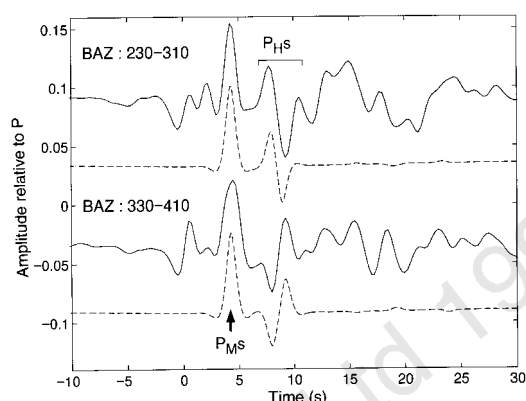


Figure 4 Composite simultaneously deconvolved, radial seismograms (solid lines) along two back azimuthal corridors ($240\text{--}310^\circ$ and $330\text{--}410^\circ$). Dashed lines are corresponding reflectivity synthetic seismograms for a model comprising isotropic crust overlying isotropic mantle with embedded orthorhombic H layer exhibiting $\pm 5.5\%$ S -wave velocity anisotropy along the z -axis, using the formulation of Martin and Thomson.²⁹ The reverberations P_{MS} , P_{HS} which arrive after 14 s, well after P_{HS} , have not been included in the synthetics. Note the reversal in polarity of P_{HS} between the two corridors which occurs at about 315° back azimuth. A phase shift of $\sim 45^\circ$ with back azimuth exists between polarity reversals on the radial and transverse components. This cannot be explained by an isotropic, dipping layer but, rather, signals the presence of anisotropy. Note that this manifestation of anisotropy, the reprojection of particle motions across interfaces to satisfy boundary conditions,³⁰ is distinct from the splitting of, for example P_{MS} through an anisotropic crust, as has been documented in previous work.³¹

different and allow for an H that reflects a fossilized fabric developed in an ancient thermal regime. It remains to be seen whether either mechanism is capable of producing the interface transition widths of < 200 m required by the seismic reflection data¹¹. A third possibility which may better accommodate this last constraint involves a structural origin perhaps associated with craton assembly through shallow subduction, imbrication and underplating.^{21–23} This mechanism permits compositional contributions to layering which may be required in order to explain the multilayer origin inferred for the reverberatory P_{135S} phase. Note, however, that lower levels of shear velocity anisotropy in eclogite may render it a less likely candidate for H than peridotite²⁴.

The observation of a simpler L interface which increases in depth away from the craton margin suggests a relation with the boundary separating cold, coarse harzburgites and hot, sheared lherzolites in South Africa, which varies in depth according to age of overlying crust²⁵. Arguments for an explanation of L in terms of anisotropy are well established^{26–28} although the data presented here cannot easily be used to distinguish between the effects of anisotropy and lateral heterogeneity for this interface. The absence of prominent, strongly coherent arrivals below L (Fig. 3) does, however, support the notion that L separates a mechanical boundary layer from more isotropic underlying mantle^{27,28}. A more comprehensive assessment of this and other issues raised herein will be possible as results from a larger body of stations are analysed and depth, strength, orientation and tectonic affinity of these shallow mantle boundaries are documented. □

Received 3 April; accepted 1 October 1997.

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Acknowledgements. I am grateful to Colin Thomson for providing notes and algorithms on wave propagation in anisotropic media, and to the Geological Survey of Canada for allowing access to the Yellowknife array data archives. Reviews by James Gaherty, Harold Gurrilla, and Paul Silver improved the final manuscript. This work was supported by BHP, Lithoprobe, Monopros and NSERC.

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Test of synergistic interactions among deleterious mutations in bacteria

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Identifying the forces responsible for the origin and maintenance of sexuality remains one of the greatest unsolved problems in biology^{1–6}. The mutational deterministic hypothesis postulates that sex is an adaptation that allows deleterious mutations to be purged from the genome; it requires synergistic interactions, which means that two mutations would be more harmful together than expected from their separate effects^{4,5}. We generated 225 genotypes of *Escherichia coli* carrying one, two or three successive mutations and measured their fitness relative to an unmutated competitor. The relationship between mutation number and average fitness is nearly log-linear. We also constructed 27 recombinant genotypes having pairs of mutations whose separate and combined effects on fitness were determined. Several pairs exhibit

significant interactions for fitness, but they are antagonistic as often as they are synergistic. These results do not support the mutational deterministic hypothesis for the evolution of sex.

Within a population of organisms, an asexually reproducing genotype should have twice the fitness of its sexual counterparts, all else being equal^{1,2}. Therefore, it is difficult to understand why sexual reproduction is so widespread in nature³. This problem remains unsolved not for want of theories, but rather for lack of compelling data. Among some 20 hypotheses⁵, two postulate that sex is an adaptation for purging deleterious mutations from the genome^{4–6}. One of these two, Muller's ratchet^{7,8}, depends on random genetic drift and thus provides an advantage for sex only in small populations. In contrast, the mutational deterministic hypothesis^{4,5} is effective in large populations but requires synergistic epistasis, such that multiple mutations are typically more harmful together than would be expected from their separate effects (Fig. 1). In mathematical form, let

$$\ln W_k = -\alpha k - \beta k^2,$$

where W_k is the average fitness of genotypes with k mutations, $\alpha > 0$ for deleterious mutations, and β defines the interaction between mutations. The intercept is fixed at zero because fitness values are expressed relative to an unmutated progenitor. If mutations interact synergistically ($\beta > 0$), then the equilibrium load of deleterious mutations is lower in a sexual than in an asexual population^{9,10}, providing an advantage for sex. (This requirement for synergistic epistasis has recently been extended to bacterial transformation, a form of non-mendelian sex¹¹.)

There are few data to indicate whether deleterious mutations typically interact in a synergistic manner, and previous attempts to address this issue have been hampered by methodological limitations. For example, some studies have examined interactions between whole chromosomes isolated from nature^{12,13}, whereas others allowed spontaneous mutations to accumulate by population bottlenecks^{14,15} or have used mutagens to achieve the same effect^{13,16}; these approaches all depend on indirect estimates of mutation number in experimental genomes. Other studies have examined interactions among defined sets of mutations¹⁷, which may not be representative of the whole genome. There is the added problem that genomes with several mutations may be more difficult to obtain, and hence to measure, than genomes with only one mutation. Also, most studies have relied on incomplete fitness estimates in organisms with complex life-cycles, such as fruitflies. Moreover, dominance and inbreeding effects may obscure interactions in diploid organisms¹⁸. Finally, a recent experiment with

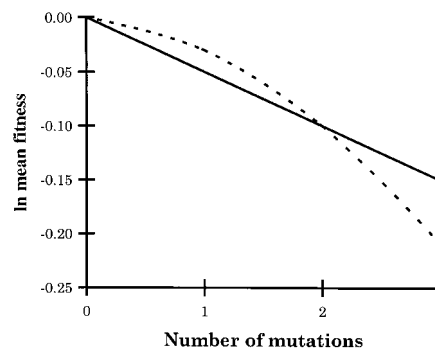


Figure 1 Hypothetical effects of increasing numbers of deleterious mutations on fitness. Each curve is based on the general model given in the text. The solid line illustrates multiplicative effects ($\alpha = 0.05$; $\beta = 0$); each additional mutation causes a comparable reduction in fitness. The dotted curve shows synergistic epistasis ($\alpha = 0.01$; $\beta = 0.02$); each additional mutation causes a disproportionate fitness reduction. The mutational deterministic hypothesis for the evolution of sex requires synergistic interactions among deleterious mutations.

yeast suggests that sex provides an advantage by the elimination of deleterious mutations, but it cannot distinguish between the effects of Muller's ratchet and synergistic epistasis¹⁹. Our approach avoids these methodological problems.

The most direct approach for determining the relationship between mutation number and fitness is to construct genotypes with different numbers of random mutations and measure their relative fitness. The bacterium *E. coli* provides an excellent system for this approach. The particular strain we used was sampled from a population that was propagated in a defined environment for 10,000 generations, during which time its rate of evolutionary adaptation slowed dramatically²⁰. Hence, this strain was near a selective optimum in that environment. Three mini-Tn10 transposons, each having a different antibiotic-resistance marker, were used to generate insertion mutations²¹. These transposons had been modified so that the site of insertion was effectively random²¹. We identified mutants under permissive conditions, on a nutritionally rich medium; we then measured their fitness under stringent conditions, in the same minimal medium in which their progenitor evolved. Hence, the sample is unbiased with respect to recovery of mutations, and fitness is expressed in the same environment as the organism's recent selective history. We constructed 225 genotypes, including 75 each with one, two or three insertion mutations. Each numerical class was balanced with respect to the three resistance markers. For every genotype, we performed at least three competition assays, which typically ran for 40 generations, to measure fitness relative to an unmutated genotype. Relative fitness is simply defined as the ratio of the net growth rates of the mutated and unmutated genotypes during competition for limited resources.

Figure 2 shows the relationship between mutation number and average fitness. A log-linear regression explains more than 99% of the variation in sample means ($F_{1,2} = 2,864.6$, $P = 0.0003$, $r^2 = 0.9993$). Inclusion of a quadratic term, as postulated by the mutational deterministic hypothesis, does not significantly improve the model's fit to the data ($F_{1,1} = 1.4353$, $P = 0.4428$). To maximize statistical power, one would like to perform these regressions using each mutant as an independent observation. In practice, this approach introduces technical difficulties because: (1) several mutants have zero fitness, so that the logarithm is undefined; and (2) the variance in fitness increases with mutation number, which violates an assumption of least-squares regression. To circumvent these problems and achieve statistical power commensurate with a sample of 225 genotypes, we used a robust method called Tukey's jack-knife²² to obtain 225 quasi-independent estimates of the linear coefficient, α , and the quadratic coefficient, β . The jack-knifed

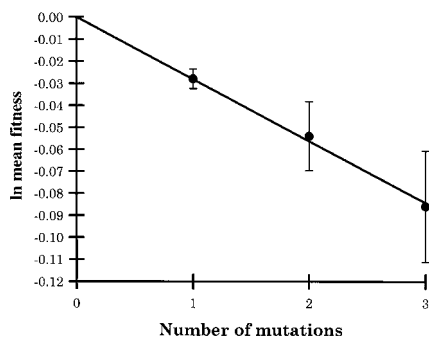


Figure 2 Observed effect of increasing the number of deleterious mutations on fitness in the bacterium, *E. coli*. Each point represents the average fitness value for 75 different genotypes carrying one, two or three insertion mutations. Error bars indicate standard errors, using the jack-knife method²². The solid line shows the best fit of a log-linear (multiplicative) model to the data. A model with synergistic epistasis, which includes an extra quadratic term, does not yield a significant improvement in the fit to the data.

estimate of α is 0.0273 (standard error = 0.0059, $t_{224} = 4.6510$, $P < 0.0001$). The corresponding estimate of β is 0.0037, which does not differ significantly from zero (standard error = 0.0070, $t_{224} = 0.5247$, $P = 0.6003$). Thus, there is no clear tendency for deleterious mutations to interact synergistically.

This finding is a negative result, and the objection can be raised that our experiment lacked sufficient power to reject the null hypothesis. Statistical power reflects several factors, including sample size and the ratio of signal to noise. Computation of variance components²² indicates that the genetic variance in fitness among the 225 mutants ($s_G^2 = 0.0204$, $F_{224,460} = 132.7$, $P < 0.0001$) was 40-fold greater than the error variance due to imprecision in measuring fitness ($s^2 = 0.0005$). Thus, the negative result cannot be due to noisy measurements. More problematic for resolving the shape of the fitness function is the existence of substantial fitness variation among genotypes within each numerical class, which is a fact of life (but one ignored in most theoretical treatments of this subject). The most useful summary of statistical power is given by the confidence limits for any parameter of interest. Using standard errors from the jack-knife analysis, the true value of β is between -0.0101 and 0.0174 with 95% confidence. (We can also use the jack-knife analysis to obtain the 95% confidence interval for the ratio β/α , which runs from -0.2100 to 0.4774.) The question is then: how large must β be for synergistic epistasis to be important with respect to the hypothetical advantage of sex? There is no easy answer to this question; it depends on other variables, including especially the genomic deleterious mutation rate^{4,5} (which can vary greatly even among genotypes of the same species²³). For example, if β is positive and not too much smaller than α (such that $\beta/\alpha = 0.4$), then the increase in mean fitness due to sex is only a few per cent when the genomic deleterious mutation rate is 0.1, but the advantage of sex is about twofold when the rate is 2.0 (ref. 24). The genomic deleterious mutation rate in *E. coli* is $< 10^{-3}$ for a genotype with functional DNA repair mechanisms²⁵, and so the benefit of sex is very slight even for the highest value of β consistent with our experiment. However, our primary objective is not to evaluate whether sex would be advantageous for *E. coli*. Rather, we seek to determine if there is a general tendency for genetic architectures to exhibit synergistic epistasis among deleterious mutations, which does not appear to be true. But given the statistical uncertainty inherent in this negative result, we conservatively summarize the regression analyses by reiterating that precise measurements of the fitness of 225 genotypes, each having from one to three mutations, were insufficient to demonstrate significant synergistic epistasis. Any comparable study that seeks to prove synergistic epistasis must

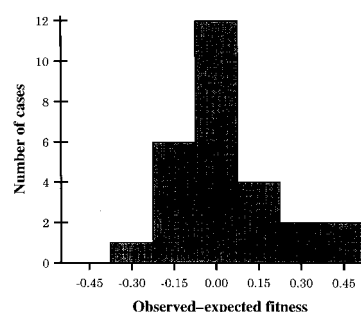


Figure 3 Distribution of observed minus expected fitness values for 27 different double mutants. The expected fitness values are calculated from fitness values of the corresponding single mutants assuming no interaction (multiplicative effects). Synergistic interactions are neither stronger nor more common than antagonistic interactions.

(1) examine many more genotypes, (2) generate a much wider range of numbers of mutations per genome, or (3) show that some other organism has a much greater tendency for synergistic epistasis than does *E. coli*.

A roughly log-linear fitness function has two possible explanations. One is that the effects of mutations on fitness are almost always independent. Alternatively, deleterious mutations may often interact, but synergistic and antagonistic interactions may be more or less equally common, so that on average there is no clear deviation from log-linearity. Otto and Feldman²⁶ recently showed theoretically that "variability among loci in the sign and strength of epistasis ... decreases the parameter space over which increased recombination may evolve" and noted that "no data currently exist on the variance in epistatic interactions among random mutations." Therefore, we devised a second experiment to test these alternative explanations by examining the variability in the sign and strength of interactions among deleterious mutations.

In our first experiment, genotypes with multiple mutations were produced by successive rounds of mutagenesis. By contrast, in this second experiment, we constructed genotypes with multiple mutations by recombining mutations from genotypes that had single mutations. Thus, we could measure the effect of each particular mutation alone and in combination with other mutations. We chose three mutations, which had a broad range of fitness effects, from each of the three sets of genotypes with single mutations. We used P1 transduction (see Methods) to construct all 27 combinations of two mutations from different sets. The fitness values for each double mutant and its corresponding single mutants were measured simultaneously, with 10-fold replication each. The paired fitness values for two single mutants were used to generate the expected fitness values for the double mutants, assuming no interactions (multiplicative effects).

Figure 3 shows the distribution of observed minus expected fitness values for the 27 double mutants. A skewness test²² indicates that the distribution is effectively symmetrical ($t_{26} = 1.5336$, $P = 0.1372$); synergistic interactions are neither stronger nor more common than antagonistic interactions. For each double mutant, we ran a paired t -test of the difference between observed and expected values. Seven cases exhibit synergistic epistasis, whereas seven others indicate antagonistic interactions (all $P < 0.05$). Because we performed 27 tests for interactions, and not all were independent (using the same nine mutations), we then applied the Bonferroni method²⁷ to adjust significance levels for the multiplicity of tests. Even with this conservative approach, three synergistic and four antagonistic interactions are significant. Therefore, the mutational deterministic hypothesis seems to fail not because interactions between deleterious mutations are very rare, but rather because synergistic and antagonistic interactions are both common.

Note added in proof. If fitness is measured as a relative rate, as in our study, then an additive null model may be more appropriate theoretically for testing the mutational deterministic hypothesis (S. P. Otto, personal communication), whereas the multiplicative model is more plausible biologically. In fact, the statistical results for both experiments reported here are nearly identical using additive and multiplicative models, with the additive model yielding even less support for the mutational deterministic hypothesis. □

Methods

Transposon mutagenesis. Insertion mutations were generated using mini-Tn10 constructs and methods described in ref. 21. Briefly, transposase genes are not carried by the mini-Tn10 but are encoded by the delivery vector (a defective λ phage), so that secondary transpositions cannot occur. Also, the transposase genes have been engineered to yield insertions that are effectively random with respect to target sequence²¹. Southern blots confirmed that insertions were well dispersed through the genome; there were few, if any, identical insertions among 60 mutants screened²⁸. The three mini-Tn10 constructs used here

encode resistance to chloramphenicol, kanamycin or tetracycline. The site of an insertion mutation, and not the associated resistance marker, is primarily responsible for its fitness effect²⁸. Mutants were selected on TA agar²⁹ supplemented with antibiotic at the appropriate concentration²¹. Plates were incubated for 72 h, after which no additional colonies were observed; mutants were chosen at random with respect to their time of appearance²⁸.

P1 transduction. Recombinant genotypes that had two particular mutations were constructed by transduction using P1vir and the method described in ref. 30. Recombinants were selected on TA agar containing the relevant antibiotics. Each mutation's presence was confirmed by a Southern blot (using the resistance genes as probes), in which single and double mutants were run on the same gel. As a control to test for any effect of the transduction procedure on fitness, we performed four separate transductions to generate each of three double mutants; we measured the fitness of each construct with fivefold replication. A nested ANOVA confirms that the three double mutants have different fitness values ($F_{2,9} = 307.1$, $P < 0.0001$) whereas independent constructions of the same double mutant have the same fitness ($F_{9,48} = 0.6704$, $P = 0.7312$).

Culture conditions and fitness assays. The fitness of each mutant genotype was measured relative to a common competitor at 37 °C in DM broth²⁹, in which ammonium and glucose provide nitrogen and carbon, respectively. To distinguish competitors in the fitness assays, we used a common competitor²⁸ that was Ara⁺, whereas all the mutants were Ara⁻; the common competitor and mutants produce white and red colonies, respectively, on TA agar²⁹. We ran 195 controls using the unmutated Ara⁻ progenitor and the Ara⁺ common competitor; the fitness for each mutant was then standardized relative to the average value of these controls²⁸. Before each fitness assay, the two competitors were separately grown in DM to acclimatize them to the experimental conditions. They were then each diluted 200-fold into the same fresh DM broth and a sample was immediately plated on TA agar to estimate their initial densities. Every day, for six days, the mixed population was diluted 100-fold into fresh DM; the population then grew 100-fold, or ~ 6.6 ($= \log_2 100$) cell generations, after which the glucose was depleted. After six days, a sample was plated on TA agar to obtain each competitor's final density. Some mutants had such low fitness that their final frequencies were below 5%; for those mutants, we reran the competitions for three days (or, in a few cases, one day) to ensure sufficient numbers in the final samples to obtain accurate fitness estimates. For each competitor, we calculated its growth rate as $m = \ln(100 \cdot N_f/N_0)/t$, where N_0 and N_f are initial and final densities, respectively, and t is the number of days of competition. (All densities are expressed before dilution; the factor of 100 reflects the 100-fold dilution and re-growth each day.) The relative fitness of two genotypes is defined as the ratio of their growth rates during competition for the same pool of nutrients²⁹. This fitness measure thus reflects differences in reproduction and survival during every phase (lag, growth and stationary) of the serial batch regime²⁹.

Received 27 May; accepted 22 August 1997.

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Acknowledgements. We thank V. de Lorenzo, L. Forney, J. Peck and O. Schabenberger for advice and encouragement; L. Eklunwe and N. Hajela for technical support; and A. de Visser, J. Mongold, P. Moore, S. Otto, B. Rice, D. Rozen and C. Zeyl for valuable comments. This work was supported by a fellowship from the Spanish MEC to S.F.E. and a grant from the NSF to R.E.L.

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Antisaccade performance predicted by neuronal activity in the supplementary eye field

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The voluntary control of gaze implies the ability to make saccadic eye movements specified by abstract instructions, as well as the ability to repress unwanted orientating to sudden stimuli. Both of these abilities are challenged in the antisaccade task, because it requires subjects to look at an unmarked location opposite to a flashed stimulus, without glancing at it^{1,2}. Performance on this task depends on the frontal/prefrontal cortex and related structures^{3–8}, but the neuronal operations underlying antisaccades are not understood. It is not known, for example, how excited visual neurons that normally trigger a saccade to a target (a prosaccade) can activate oculomotor neurons directing gaze in the opposite direction. Visual neurons might, perhaps, alter their receptive fields depending on whether they receive a pro- or antisaccade instruction. If the receptive field is not altered, the antisaccade goal must be computed and imposed from the top down to the appropriate oculomotor neurons. Here we show, using recordings from the supplementary eye field (a frontal cortex oculomotor centre) in monkeys, that visual and movement neurons retain the same spatial selectivity across randomly mixed pro- and antisaccade trials. However, these neurons consistently fire more before antisaccades than prosaccades with the same trajectories, suggesting a mechanism through which voluntary antisaccade commands can override reflexive glances.

In an antisaccade, a single visual stimulus acts both as a landmark for and a distractor from the goal of the required movement. The need to use the target location conflicts with the interdiction to glance at it. If a powerful reflex attracts gaze to the target, or at least draws attention to it⁹, how can it be repressed? The answer depends on how the antisaccade is programmed by the brain. We know that each cerebral hemisphere contains: visual neurons that respond to stimuli appearing in one visual hemifield, at specific locations called

receptive fields; movement neurons activated shortly before saccades directed towards subregions of the same hemifield, called movement fields; and visual-and-movement neurons that have both a receptive field and a movement field, usually in close spatial correspondence when prosaccades are generated. We began by considering the hypothesis that the instruction to make an antisaccade causes receptive fields to shift from one hemifield to the other so that both the visual neurons and the appropriate movement neurons they would activate after the shift would be in the same hemisphere. The initial reflex, no longer sustained by visual activity in the opposite hemisphere, would be short-lived or non-existent. We know that receptive-field shifts are possible as they have been observed in other experiments¹⁰. Alternatively if visual receptive fields do not move, the antisaccade goal must be calculated and imposed on the neurons that have the appropriate movement field. Under this hypothesis, the flashed stimulus would give rise to concurrent streams of neuronal signals; one, if dominant, would trigger a reflexive prosaccade, and the other would initiate the correct antisaccade. The conflict of neuronal signals thus created would have to be resolved at a 'winner-take-all' junction in the brain, where the strengths (for example, the intensity of firing) of different saccadic commands are weighed and the strongest one is translated into behaviour. The 'winner-take-all' principle has been found to account for perceptual decisions¹¹. It should be noted, however, that if the rule 'one neuron, one vote' applies, the odds of winning are very poor for antisaccade commands, given the ubiquity of neurons that discharge more strongly before saccades to visual targets than before self-initiated saccades.

We searched for evidence supporting the first hypothesis (the shift in receptive field), in the two oculomotor centres found indispensable for performing antisaccades: none were found in either the frontal eye field (FEF)¹² or the supplementary eye field (SEF). However, we observed that neuronal discharges in the SEF were consistently greater before antisaccades than before prosaccades with the same trajectories. This bias could be the means by

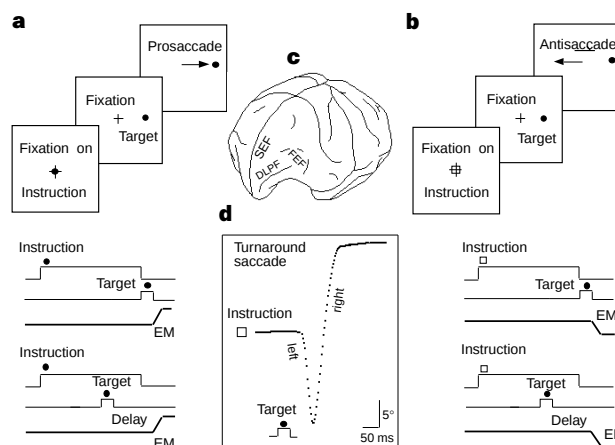


Figure 1 Spatial display and sequence of stimuli and saccades. **a**, Prosaccade trial; **b**, antisaccade trial. Top, the spatial display of stimuli and saccades; bottom, the traces indicate the sequence of events. Each trial began with a fixation point stimulus encoding, by its shape, the instruction to make a prosaccade (small dot) or an antisaccade (small square). The disappearance of the fixation point was the trigger signal for the eye movement (Em). An eccentric visual stimulus (always a dot) was flashed for 50–100 ms when the fixation point disappeared (trigger signal for the saccade) or at variable times during the fixation of the instruction cue. **c**, Location of the frontal eye field (FEF), supplementary eye field (SEF), and dorsolateral prefrontal cortex (DLPF). **d**, Sample record of an occasional 'turnaround saccade' occurring on an antisaccade trial. The first saccade was directed in error to the visual stimulus, then, without a pause, the correct antisaccade followed.

which the brain offsets the tendency to respond reflexively to a sudden stimulus. As for the existence, at any time, of a covert parallel processing of saccades in the opposite direction¹³, it was attested by the occasional occurrence of turnaround saccades (Fig. 1) at any latency from stimulus onset. The time interval between such saccades is too short to assume that they are prepared in succession.

Here we concentrate on results obtained in the SEF which, like the FEF, contains visual, visual-and-eye movement, and movement neurons^{14,15}, produces saccades on electrical stimulation¹⁴, and has direct pathways to the brainstem oculomotor apparatus¹⁶. The SEF also contains neurons activated before self-initiated saccades in darkness¹⁴, neurons activated when the monkey is instructed to withhold a saccade¹⁵, and neurons that modify their response patterns under oculomotor conditioning¹⁷.

Two rhesus monkeys were trained to perform interleaved pro- and antisaccades (Fig. 1a, b) in eight general directions, allowing us to test SEF neurons by presenting a target at sites evoking their greatest visual or motor responses. Each cell was tested on four types of trials. On prosaccade trials (see white boxes in Figs 2 and 3), the

visual target and the saccade goal were both in the centre of conventionally mapped receptive fields and movement fields (yy, yes–yes trials) or they were not (nn, no–no trials). In antisaccade trials (grey boxes in Figs 2 and 3), the target and the saccade were necessarily mismatched: either the visual target was in the receptive field (yn trials), or the saccade was in the movement field (ny trials). We studied a subset of 39 cells exhibiting visual or movement activation or both. No shift of visual or movement field was observed. The field stability across pro- and antisaccade trials was most striking in visual-and-movement cells when a delay was imposed between the flashed target and the trigger signal for the saccade. Thus the cell illustrated in Fig. 2a gave visual responses, time-locked to stimuli, regardless of the saccade direction, that is, on yy and yn trials. It also showed an activation time-locked to the appropriate eye movement, regardless of the stimulus location, that is, on yy and ny trials. This pattern of firing excludes the first hypothesis, that the receptive fields shift.

The tendency of neurons to fire significantly more ($P < 0.05$) before antisaccades than before prosaccades in its movement field (compare yy and ny trials in Fig. 2a, b illustrating a movement-only cell) fits with the assumption of a competition to be won at a 'winner-take-all' junction. Furthermore, on antisaccade trials yn, when the monkey made a prosaccade in the movement field by mistake, the firing rate was often significantly lower, becoming similar to that associated with correct prosaccades (Fig. 2c). This suggests that the relative increase of firing on correct antisaccade trials was needed to win the competition.

We then examined how early the level of activation of SEF neurons differentiates antisaccades from prosaccades in the sensorimotor transformation occurring during a trial. This question bears on the potential role of the SEF in the inhibition of quick reflexive glances. For some cells, a significant difference between pro- and antisaccade trials had already appeared at the time of visual

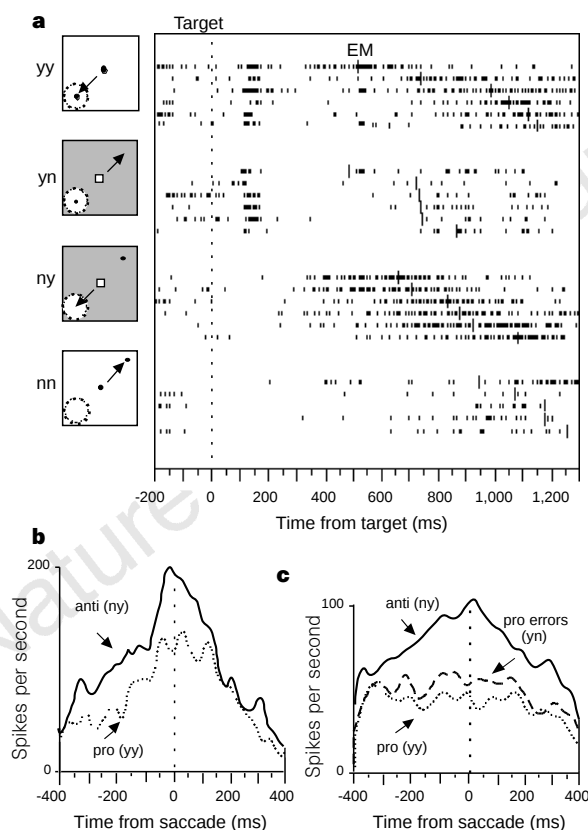


Figure 2 Performance of delayed saccades and activity profiles of pro- and antisaccades. **a**, Typical visual-and-movement cell studied during the performance of delayed saccades, in four types of trials shown in white (prosaccades) and grey (antisaccades) boxes to left of the rasters of action potentials. Symbols in boxes: dot, prosaccade instruction; square, antisaccade instruction; eccentric dot, visual stimulus; arrow, saccade; dotted circle, response field. The rasters are aligned on target onset and rank ordered according to saccade onset (vertical bars). Maximum activity occurred with stimuli and saccades of 20° down-left. **b**, Same cell, showing activity profiles of antisaccades (solid line) and prosaccades (dotted line), aligned on saccade onset (at 0 ms). The activity preceding the movement was significantly higher for antisaccades than for prosaccades ($P < 0.05$). **c**, A movement-only cell showing, as in **b**, a significant difference between correct anti- and prosaccades. In addition, a significant difference existed between correct antisaccades (solid line) and prosaccades made in error (dashed line).

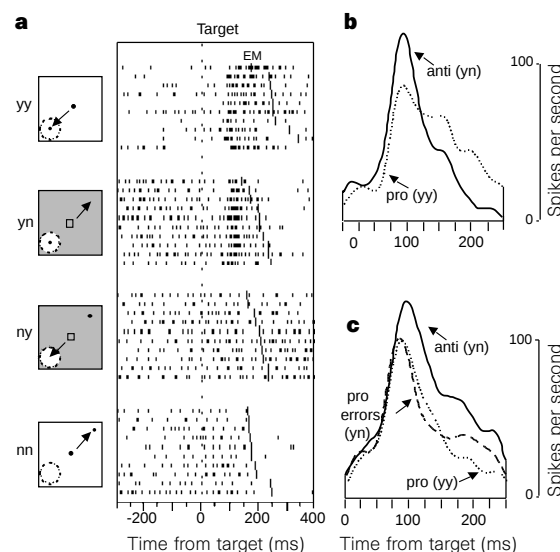


Figure 3 Performance of non-delayed saccades and activity profiles of pro- and antisaccades. **a**, Visual cell studied during the performance of non-delayed behaviour, in the four types of trials shown in white (prosaccades) and grey (antisaccades) boxes. Symbols in boxes are as in Fig. 2. The rasters are aligned on the onset of the target and rank ordered according to saccade onset (vertical bars). This cell was silent between trials, and started firing after the fixation point appeared, about 600 ms before the target. **b**, Comparison of activity profiles of antisaccades (solid line) and prosaccades (dotted line) aligned on target onset (at 0 ms). Visual responses were significantly greater on antisaccade trials ($P < 0.05$). **c**, Another visual cell showing the greatest responses with antisaccades (solid line) and significantly smaller responses with correct (dotted line) and mistaken (dashed line) prosaccades.

responses (Fig. 3). Like presaccadic cells, some visual cells seemed to predict the occurrence of correct antisaccades by their stronger responses compared with those occurring before correct or incorrect prosaccades (Fig. 3c). It should be noted that, although the visual target was identical on anti- and prosaccade trials, its behavioural significance had already been cued by the previously given instruction, perhaps warning the monkey against premature prosaccades.

The distribution of movement cells and visual cells, according to their differential pro-versus antisaccade activity (Fig. 4a), shows that the neurons having the properties described above (Figs 2 and 3) represent a majority in the SEF. The group profiles of firing rate (Fig. 4b) corroborate the observations made on individual neurons; examples are given for movement cells (ny/yy) and for visual cells (yn/yy). The group profiles also show: that when visual cells responded differentially on pro- and antisaccade trials, they did so sharply; and that the higher level of activity on antisaccade trials was continuous and already present before the direction of the movement was specified. To determine whether this is an effect of the instruction cue given during the fixation period, thus revealing a top-down control on the selection of a motor response only partly informed by an initial cue¹⁸, future antisaccade experiments should focus on SEF cells active during fixation of the instruction cue¹⁹. A top-down control may be necessary to block the occurrence of these extremely short-latency saccades, called express saccades, which can be made only reflexively to a visual target²⁰. Significantly, the only express saccades produced by our monkeys were prosaccades made in error (N.A. *et al.*, manuscript in preparation).

Probing the possible mechanisms of antisaccade generation, we were able to eliminate the hypothesis of a shift in receptive field. In contrast, we found evidence for the 'winner-take-all' principle. This evidence rests on the higher activity observed before antisaccades compared with prosaccades, and the lower activity observed when prosaccades are made in error. It is possible for antisaccade commands to prevail by a shorter latency of response, a larger number of involved neurons, or a greater intensity of firing. Our results favour the last possibility but do not exclude the others.

Successful performance of antisaccades is not likely to depend on the SEF alone. Brain imaging^{5,6} in humans has implicated three frontal cortical centres: the dorsolateral prefrontal cortex (DLPF), the primary frontal eye field (FEF), and the SEF, each of which contributes to antisaccade initiation but probably in a different way. A pioneer study of the DLPF has shown that all of the signals necessary for programming an antisaccade can be encoded in the prefrontal working memory⁸. The FEF, mostly known for its

prominent role in prosaccade initiation^{21–24}, also exerts a direct inhibitory control on its counterpart in the other hemisphere²⁵ and on the superior colliculus²⁶, which is necessary for the generation of express saccades²⁷. The intervention of the SEF may be needed because the antisaccade is a willed movement to an internally defined target²⁸. However, it is not known how the brain computes an inverted vector (the metrics of the antisaccade). □

Methods

Two rhesus monkeys implanted with scleral search coils learned to perform interleaved pro- and antisaccades of varying amplitudes (10–25°) in eight directions. Dot stimuli (0.3°) could be presented anywhere on a tangent screen; their precise location during recording sessions was made to coincide with the centre of receptive field or movement field of each cell. The monkey was rewarded by a drop of juice when pro- or antisaccades terminated within an electronic window of diameter 10° for a 20° saccade. The temporal sequence of events is described in Fig. 1 for two versions of the task: immediate pro- or antisaccades (top traces), and delayed saccades obtained by manipulating the timing of the trigger signal (bottom traces). Recordings from 245 neurons were made in four SEF areas^{14,15,16,29,30}, identified by low-threshold microstimulation (for example, 30 μ A) and histological examination (one monkey). Electrophysiological methods have been described previously¹⁴. Spike trains were analysed off-line and convolved with gaussian filters (routinely, $s = 20$ ms; the profiles of activity in Figs 2 and 3 were smoothed with $s = 30$ ms to illustrate their main trend.) The statistical significance of differences between spike counts obtained on pro- and antisaccade trials for identical stimuli or identical saccades was evaluated by *t*-tests for unequal pairs, $\alpha = 0.05$. For comparing motor activity, spike counts were made in a time window routinely extending from –300 to 0 ms (0 ms indicates saccade onset) for delayed saccades, and –150 to 0 ms for non-delayed saccades. For comparing visual responses, the time window routinely extended from target onset (0) to +150 and +250 ms.

Received 4 November 1996; accepted 21 October 1997.

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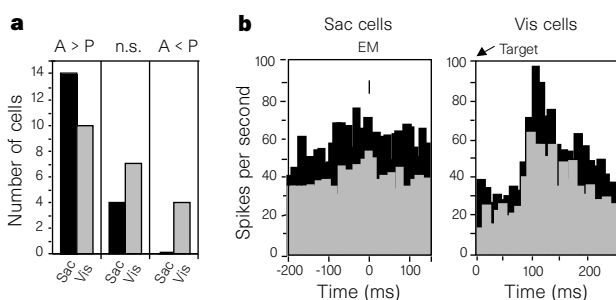


Figure 4 Activity of antisaccades and prosaccades in movement cells and visual cells. **a**, Number of movement cells (Sac, black) and visual cells (Vis, grey) for which the activity was significantly greater ($A > P$), smaller ($A < P$), on anti-saccade trials than prosaccade trials ($P < 0.05$) or not significantly different (n.s.). **b**, Each histogram represents the compound activity of antisaccade (black) and prosaccade (grey) trials, respectively, for the movement cells (Sac) and visual cells (Vis) represented in **a**. The trials compared are ny versus yy for movement cells, and yn versus yy for visual cells.

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Acknowledgements. We thank P. Dassonville, A. Pouget and J.-R. Tian for comments and suggestions, and the National Eye Institute for financial support.

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Nicotine activates and desensitizes midbrain dopamine neurons

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Tobacco use in developed countries is estimated to be the single largest cause of premature death¹. Nicotine is the primary component of tobacco that drives use, and like other addictive drugs, nicotine reinforces self-administration and place preference in animal studies^{2–5}. Midbrain dopamine neurons normally help to shape behaviour by reinforcing biologically rewarding events, but addictive drugs such as cocaine can inappropriately exert a reinforcing influence by acting upon the mesolimbic dopamine system^{3–6}. Here we show that the same concentration of nicotine achieved by smokers activates and desensitizes multiple nicotinic receptors thereby regulating the activity of mesolimbic dopamine neurons. Initial application of nicotine can increase the activity of

the dopamine neurons, which could mediate the rewarding aspects of tobacco use. Prolonged exposure to even these low concentrations of nicotine, however, can cause desensitization of the nicotinic receptors, which helps to explain acute tolerance to nicotine's effects. The effects suggest a cellular basis for reports that the first cigarette of the day is the most pleasurable, whereas the effect of subsequent cigarettes may depend on the interplay between activation and desensitization of multiple nicotinic receptors⁵.

A common feature of addictive drugs such as cocaine, amphetamine, morphine and nicotine is that at the doses at which they are self-administered those drugs increase the concentration of dopamine in the nucleus accumbens (NAcc)^{3–7}. The main dopaminergic projections to the NAcc arise from ventral tegmental area (VTA) neurons of the mesolimbic dopamine system. *In vivo* studies have shown that nicotine self-administration is reduced by lesions of this mesolimbic pathway or by nicotinic antagonists micro-infused into the VTA^{8,9}. Our study explores the cellular basis for these *in vivo* observations.

VTA neurons express nicotinic acetylcholine receptors (nAChRs), as has been shown by autoradiography for ligands and *in situ* hybridization for specific nicotinic subunits^{10–12}. Previous work has also shown that high concentrations of nicotine can depolarize and activate dopaminergic neurons¹³. There are no results, however, in the range of nicotine concentrations achieved by smokers. A smoker's level of nicotine reaches about 0.5 μM immediately after smoking a cigarette; 0.1 to 0.5 μM is the general range achieved by smokers, a range that has been shown in animal studies to have addictive power^{7–9,14}. We have found that such low levels of nicotine can have multiple effects on VTA dopaminergic neurons.

Figure 1a shows a biocytin back-filled VTA neuron from a rat brain slice, and Fig. 1b shows the same neuron labelled for tyrosine hydroxylase (TH), an enzyme for catecholamine (dopamine) synthesis. That same neuron (Fig. 1c), and all the neurons we studied, displayed an inward cationic current (I_h), which is indicative of dopaminergic neurons¹⁵. Figure 1d illustrates the general experimental setup for whole-cell voltage or current clamping of a VTA neuron from the slice. In current-clamp mode, 0.5 to 0.1 μM nicotine applied to the whole bath depolarized the neuron and caused spontaneous firing of action potentials (Fig. 1e, f, upper). When bath-applied, 0.1 μM nicotine produced a 4.3 ± 0.2 mV

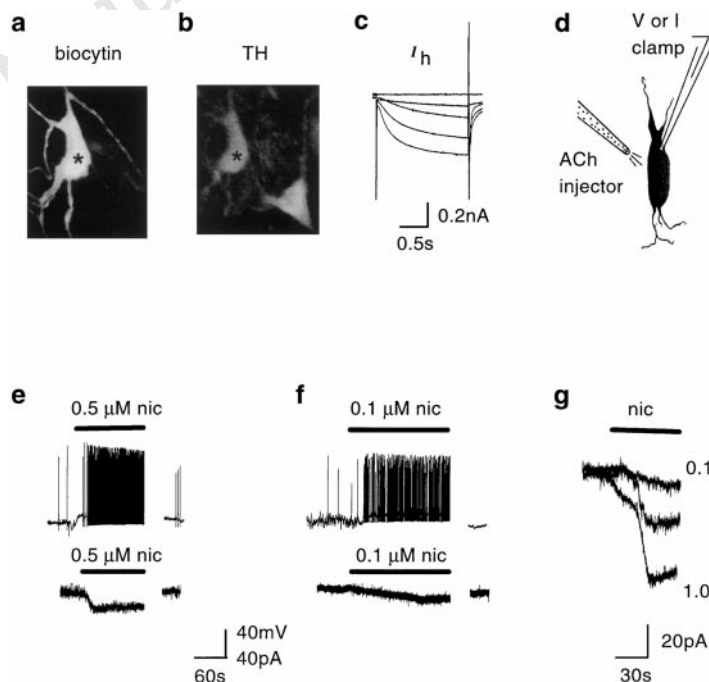


Figure 1 Dopaminergic neurons respond to nicotine. **a**, A biocytin back-filled neuron from the VTA. The neuron was visualized with fluorescein-conjugated avidin. **b**, The same neuron (*) and a nearby neuron were labelled with a monoclonal anti-TH antibody that was visualized with a Texas red-conjugated IgG. **c**, The I_h current is shown for the same neuron as in **a** and **b**. The neuron was voltage-clamped at a holding potential of -60 mV, and hyperpolarizing voltage steps of -10 mV were applied (-60 to -100) to activate the inward-going current indicative of dopaminergic neurons. **d**, Schematic drawing of a whole-cell clamped dopaminergic neuron in the VTA slice. **e**, Current-clamp recording (above) from a VTA neuron. Bath-application of 0.5 μM nicotine (solid bar) caused a 12 mV depolarization and a burst of action potentials. The record is shown with a 10 min gap during the washout of nicotine. In voltage-clamp mode (below), 0.5 μM nicotine in the bath caused a 27 pA inward current. There is a 5 min gap during washout. **f**, Current-clamp (above, 5 mV depolarization) and voltage-clamp (below, 16 pA current) recordings in response to 0.1 μM nicotine applied to the bath. The neurons generally responded more slowly to lower agonist concentrations. **g**, Example currents induced by 0.1, 0.5 and 1.0 μM nicotine applied to different neurons.

($n = 6$, s.e.m.) depolarization and $0.5 \mu\text{M}$ produced $8 \pm 1 \text{ mV}$ ($n = 12$). In voltage-clamp mode (Fig. 1e, f, lower), an inward current activated by bath application of nicotine was revealed as the underlying cause of the depolarization that initiated action potentials in the VTA neurons. When bath-applied, $0.1 \mu\text{M}$ nicotine produced a $13 \pm 1 \text{ pA}$ ($n = 11$, s.e.m.) inward current, $0.5 \mu\text{M}$ produced $36 \pm 3 \text{ pA}$ ($n = 16$), and $1 \mu\text{M}$ produced $57 \pm 9 \text{ pA}$ ($n = 7$). Examples of the nicotine dose-dependence are shown in Fig. 1g.

To investigate the effect of nicotine in greater detail, the muscarinic acetylcholine receptors were inhibited and a pipette containing 1 mM ACh was placed 15 to $30 \mu\text{m}$ from the soma to allow brief, local pressure injections of ACh (Fig. 1d). The brief, local pressure pulses of ACh (arrow heads in Fig. 2a) produced inward currents. Upon applying $0.5 \mu\text{M}$ nicotine to the bath, the brief ACh-induced currents were desensitized and there was a downward shift in the baseline. The best explanation is that the local injection of ACh activated a small number of nicotinic receptors, but bath application of $0.5 \mu\text{M}$ nicotine reached all the nAChRs on the neuron. The majority of the nAChRs were desensitized, which greatly reduced the current activated locally by the ACh injection. However, a small fraction of the total number of nAChRs stochastically opened in response to the bath application of nicotine, producing a slow inward current seen as a downward shift in the baseline.

In current-clamp mode, which more closely approximates the biological situation, the pressure injection of ACh caused a burst of action potentials (Fig. 2b). Bath application of $0.5 \mu\text{M}$ nicotine (solid bar in Fig. 2b) caused a depolarization of the resting membrane potential. After 1 min in $0.5 \mu\text{M}$ nicotine, the neuron fired continuous spontaneous action potentials that were no longer

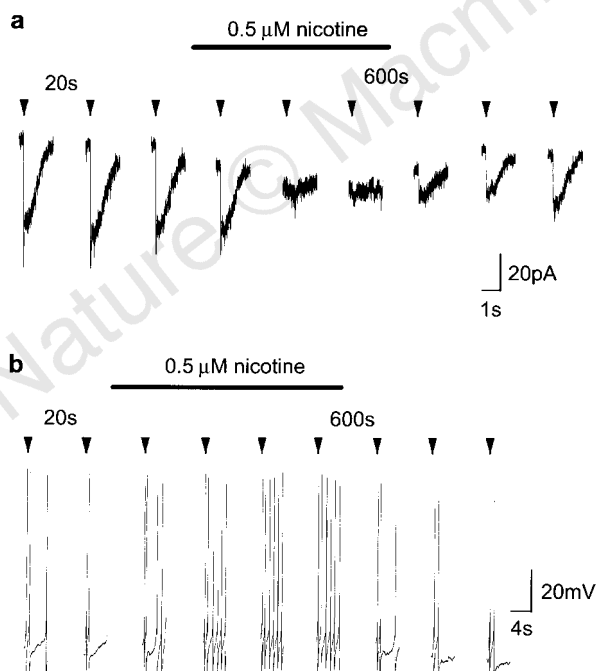


Figure 2 Bath-applied nicotine affects ACh-activated responses. **a**, When a voltage-clamped neuron experienced pressure injections of ACh (1 mM for 100 ms , arrow heads) separated by 20 s inward currents were induced. Bath application of nicotine desensitized the ACh-induced currents and induced a baseline current of 25 pA . During the washout and recovery from nicotine, ACh injections were still given every 20 s , but for clarity a 600 s gap is placed between the displayed currents. Atropine ($1 \mu\text{M}$) was present to inhibit muscarinic ACh receptors. **b**, Under current clamp, injections of ACh (1 mM for 30 ms) separated by 20 s activated action potentials. Bath-application of $0.5 \mu\text{M}$ nicotine depolarized the cell by 7 mV and produced action potentials, and the ACh injections became less effective at inducing action potentials.

influenced by the pressure injections of ACh, as would be expected if most of the nAChRs were desensitized. Recovery of the ACh-induced currents following washout of nicotine was slow, requiring nearly 30 min .

Because the half-life of nicotine in the body is about 2 h (ref. 16), smokers experience long exposures to nicotine. Therefore, we treated slices with $0.5 \mu\text{M}$ nicotine for longer times. When nicotine was applied to the bath for 19 min (Fig. 3a), initially the response was as seen previously. There was an inward current that shifted the baseline downward and the nicotinic currents caused by pressure injection of ACh were desensitized. As the nicotine application continued beyond 5 min , however, a new process was observed. In the presence of nicotine, the currents activated by pressure injection of ACh were more strongly desensitized, and the baseline current decreased nearly to its original pre-nicotine position. Similar results were found in current-clamp mode (Fig. 3b). Initially, nicotine caused the usual depolarization and consequent action potentials, but the action potentials subsequently stopped as the resting membrane potential moved toward the original pre-nicotine

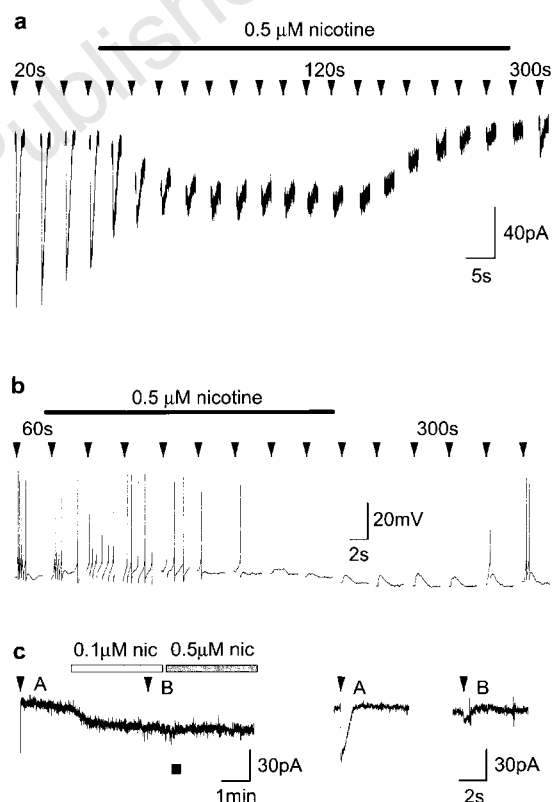


Figure 3 Longer bath applications of $0.5 \mu\text{M}$ nicotine have multiple effects. **a**, Under voltage clamp, injections of ACh (1 mM for 40 ms , arrow heads) induced inward currents that were desensitized by bath application of nicotine (solid bar). The injections of ACh were always given every 20 s , but the time interval between displayed traces is initially 20 s then 120 s and then 300 s . The current activated by bath-application of nicotine is displayed by a downward displacement of the currents induced by injection of ACh, and at later times in nicotine, the baseline current desensitizes toward its original value. **b**, Under current clamp, injection of ACh (1 mM for 40 ms) initially depolarized the neuron and caused action potentials, but at later times in nicotine the neuron did not respond to ACh. The injections of ACh were given every 20 s , but the time interval between displayed traces is altered for clarity from 60 s to 300 s . **c**, Left, bath application of $0.1 \mu\text{M}$ nicotine activated a 17 pA current. After 3 min in $0.1 \mu\text{M}$, applying $0.5 \mu\text{M}$ nicotine activated very little additional current. If there had been no desensitization, the black square marks the average size of the current activated by $0.5 \mu\text{M}$ nicotine. ACh pressure injections (1 mM , 30 ms , arrow heads) were applied before (A) and near the end (B) of the $0.1 \mu\text{M}$ nicotine. Those ACh-induced currents on an expanded time scale (right) illustrate the extent of desensitization.

value. Based on previous studies of nAChRs^{5,17–22}, a reasonable explanation is that during longer exposure to nicotine the nAChRs can enter deeper levels of desensitization, causing a greater fraction of the nAChRs to become desensitized. Thus, as the time in nicotine increases, the depolarized baseline desensitizes towards its initial resting membrane potential as fewer nAChRs are available to open stochastically to contribute to the depolarizing inward current. The rate at which the baseline depolarization recovered in nicotine toward the original pre-nicotine baseline was highly variable: $t_{1/2}$ for recovery in 0.5 μ M nicotine was 6 ± 2 min ($n = 6$). In three other experiments, the depolarization was constant and did not recover during 15-min exposure to nicotine. Figure 3c shows that even the lower concentration of nicotine (0.1 μ M) that may be maintained during much of a smoker's day can cause significant desensitization of nAChRs.

The variability in $t_{1/2}$ and in the appearance of the currents

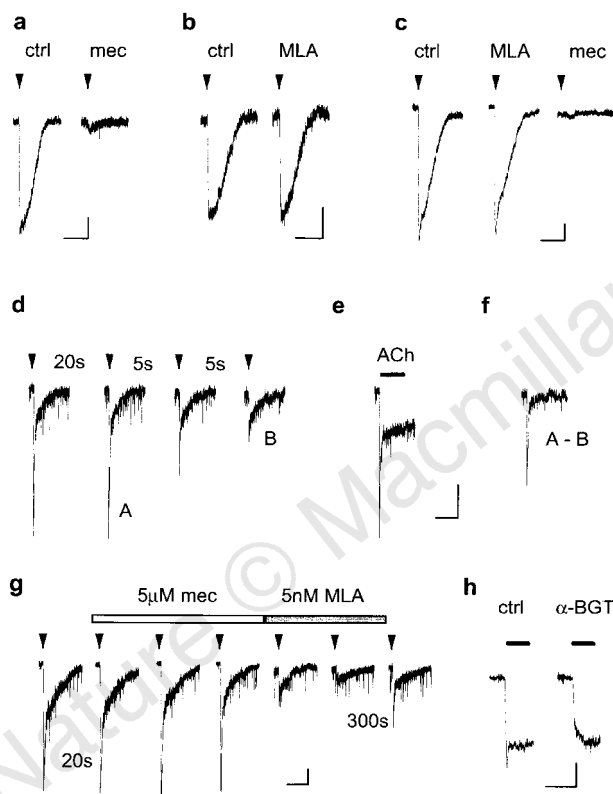


Figure 4 Pharmacology of the nicotinic currents from dopaminergic VTA neurons. All currents were activated (arrow heads) by 1 mM ACh pressure-injected onto the neurons for 30–50 ms. All scale bars are 25 pA and 1 s. **a**, ACh induced an inward current (control, ctrl) that was inhibited by bath-application of 5 μ M mecamylamine (mec). **b**, ACh induced a current (ctrl) similar to **a** that was not inhibited by bath-application of 20 nM MLA. **c**, The ACh-induced current from another neuron was not inhibited by 10 nM MLA but was inhibited by 20 μ M mec added to the bath sequentially. **d**, A more rapid ACh-induced current maintained its peak amplitude if 20 s separated ACh pressure injections. Allowing only 5 s between ACh injections caused the rapid peak of the current to desensitize. **e**, A 1 s injection of ACh (solid bar) onto the same neuron shows the 2 components of the current: a rapid peak and a more sustained component. **f**, Subtracting the currents of **d** (A – B) revealed the rapid, desensitizing component of the ACh-induced current. The currents were digitally filtered before the subtraction. **g**, The rapid peak of the ACh-induced current is not significantly inhibited by 5 μ M mec, but the peak is inhibited by 5 nM MLA. ACh injections were separated for 20 s, but a 300 s gap separates the last record that is displayed. **h**, ACh-induced (solid bars) current that has 2 components (ctrl). The fast component of the current was inhibited by 100 nM α -BGT (6 min incubation) but the sustained component was not ($n = 3$). Two currents were averaged under each condition.

induced by pressure injection of ACh suggested that there could be multiple types of nAChRs expressed on the VTA neurons^{20–22}. The various nAChR types could have different activation and desensitization characteristics. We investigated this possibility by examining the pharmacology of the currents induced by pressure injection of 1 mM ACh onto voltage-clamped VTA neurons. The majority of the neurons (15 out of 21) expressed predominantly a nicotinic current that peaked in about 50 ms, and that current was inhibited by mecamylamine but not by methyllycaconitine (MLA), a specific inhibitor of α -bungarotoxin (α -BGT)-sensitive nAChRs that contain the $\alpha 7$ subunit (Fig. 4a, b, c)^{23–25}. Other VTA neurons, however, also expressed a faster current that peaked in about 30 ms, and that current was more easily desensitized (Fig. 4d). When injections of ACh were separated by 20 s, the fast peak was maintained; but when the injections were separated by only 5 s, the fast peak desensitized but the slower component of the current remained. The two different components of the current from this neuron are seen more clearly in Fig. 4e, where a longer injection of ACh was applied. The fast current rapidly desensitized, but the slower current did not. Figure 4f shows the appearance of the fast current obtained by subtracting the slower component. MLA, the specific inhibitor of $\alpha 7$ -containing nAChRs, inhibits the fast component of the current but mecamylamine does not (Fig. 4g). Finally, the fast component is inhibited by α -BGT (Fig. 4h). The inhibition by MLA and α -BGT, the fast kinetics, and the high sensitivity for desensitization all indicate that this current is carried by $\alpha 7$ -containing nAChRs. Earlier investigations have not focused on this question, and therefore, $\alpha 7$ -containing nAChRs have not been previously detected in the VTA.

Behaviour studies indicate that nicotine is an addictive drug that reinforces self-administration, increases locomotion and reinforces place preference^{3–5,26}. Our results indicate that the concentrations of nicotine obtained by smoking can acutely excite dopaminergic VTA neurons to fire action potentials. This activity would initiate dopamine release in the NAcc and, in that way, is likely to be an important cellular mechanism for nicotine's rewarding effects^{3–7,10}. A smoker, however, maintains a rather steady low-level background of nicotine throughout the day^{5,16}, and our results indicate that longer exposures to nicotine can cause severe desensitization of VTA nAChRs. The rate of desensitization and recovery from desensitization can vary depending on which types of nAChRs are expressed on a particular neuron or in different areas of the brain. Thus, multiple phases of nAChR desensitization and recovery could underlie aspects of tolerance such that a second dose of nicotine following immediately after an initial dose does not elicit the same effects. The long half-time of nicotine and slow recovery from deep levels of desensitization may explain why many smokers report that the first cigarette is the most pleasurable of the day⁵. The nAChRs may more completely recover from long-term desensitization only after a night-time of abstinence from nicotine. Thus, the cellular mechanisms underlying some rewarding effects of nicotine and aspects of tolerance may be explained by nicotine activating and subsequently desensitizing nAChRs on VTA neurons. These results further support the hypothesis that the mesolimbic dopamine system contributes in different ways to the cellular and behavioral effects of many addictive drugs^{7,26–28}.

Methods

Midbrain horizontal slices containing the ventral tegmental area were prepared from Sprague–Dawley rats (12–25 days) using standard techniques¹³. Animal care was in accordance with institutional guidelines. Slices (200–250 μ m) were cut in cold solution (in mM, 124 NaCl, 2.5 KCl, 2.5 CaCl₂, MgSO₄, 1.25 NaH₂PO₄, 30 NaHCO₃, 2 sodium pyruvate, 10 dextrose) bubbled with 95% O₂, 5% CO₂. The slices were submerged in a continuously flowing (about 8 ml min^{–1}) bath solution at 32 to 34 °C of (in mM) 135 NaCl, 2.5 KCl, 2.5 CaCl₂, 1 MgCl₂, 21 NaHCO₃, 10 dextrose, bubbled with 95% O₂ and 5% CO₂, and unless stated otherwise 0.25–1 μ M atropine was present to inhibit

muscarinic acetylcholine receptors. Occasionally 0.5 μM tetrodotoxin (TTX) was present to inhibit action potentials. The solution in the pipette used for pressure injections contained 1 mM ACh in a bath-like solution except the buffering was with 10 mM HEPES (pH 7.3).

Neurons were visualized for patch-clamp recordings under infrared light using Nomarski optics²⁵, and to improve the optics the great majority of the slices were cut 200 μm thick. The pipette solution for voltage-clamp recordings was (in mM) 120 CsCH_3SO_3 , 10 CsCl , 10 EGTA, 5 Mg-ATP , 0.3 Na_2GTP , 10 HEPES, pH 7.3 and for current-clamp recordings half of the CsCH_3SO_3 was replaced by KCH_3SO_3 . In current-clamp mode when the internal cation was almost completely Cs^+ , the resting membrane potential underwent slow waves that led to bursts of activity²⁹. Standard patch-clamp techniques were used²⁵. The holding potential for voltage-clamp recordings was -60 or -70 mV. Currents or voltage measurements were amplified and filtered (0.2 to 1 kHz) using an Axopatch 1B with a 4-pole Bessel filter and were usually digitally sampled (1 to 5 kHz). Additional off-line filtering was used for displays. All the programs were written in Axobasic (Axon Inst.).

After recording, slices containing neurons back-filled with biocytin were fixed in 4% paraformaldehyde at 4 °C for 5 h. The slices were washed, and anti-TH antibodies (1:2,000) were added in 0.2% Triton X-100 for 24–48 h. Fluorescein-conjugated avidin (8 mg ml^{-1}) was added, and the primary TH antibody was visualized with goat anti-mouse IgG conjugated to Texas red (probes from Molecular Probes). The images' levels, brightness and contrast were adjusted with Adobe Photoshop.

The pharmacology experiments (Fig. 4) relied on pressure injection of 1 mM ACh in the presence or absence of bath-applied antagonists. Although we attempted to maximize the rate of ACh application and minimize desensitization of the $\alpha 7$ -type component, the situation in brain slices is not optimal for extremely rapid and reproducible drug or agonist applications²⁵. Thus, we expect that the rapid $\alpha 7$ -type currents may have been underestimated or not detected in some cases. Special effort is required to detect these currents. In addition, 0.5 μM mecamylamine blocked the slower component of current well, but after several minutes it would also start to inhibit the $\alpha 7$ -type currents, which is consistent with previous reports³⁰.

During longer exposures to 0.5 μM nicotine, the membrane potential first depolarized than began to recover toward the initial pre-nicotine baseline potential. A more constant level of desensitization without recovery was usually seen with 0.1 μM nicotine. To determine the $t_{1/2}$ for recovery to the initial potential, we measured the time when the maximum depolarization was first reached until the time when the potential had recovered to half of its pre-nicotine value. In 2 of the 6 cases, $t_{1/2}$ was linearly estimated from the slowly recovering membrane potential that had not yet recovered half way.

Received 15 July; accepted 22 August 1997.

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Acknowledgements. We thank Chi-feng Tseng for assistance in preparing the figures. This work was supported by the Smokeless Tobacco Research Council Inc., by the US National Institute of Neurological Disorders and Stroke and by the National Institute on Drug Abuse.

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Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance

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Acquired drug resistance is a major problem in the treatment of cancer. Of the more than 500,000 annual deaths from cancer in the United States¹, many follow the development of resistance to chemotherapy. The emergence of resistance depends in part on the genetic instability, heterogeneity and high mutational rate of tumour cells². In contrast, endothelial cells are genetically stable, homogenous and have a low mutational rate. Therefore, antiangiogenic therapy directed against a tumour's endothelial cells should, in principle, induce little or no drug resistance. Endostatin³, a potent angiogenesis inhibitor, was administered to mice bearing Lewis lung carcinoma, T241 fibrosarcoma or B16F10 melanoma. Treatment was stopped when tumours had regressed. Tumours were then allowed to re-grow and endostatin therapy was resumed. After 6, 4 or 2 treatment cycles, respectively, no tumours recurred after discontinuation of therapy. These experiments show that drug resistance does not develop in three tumour types treated with a potent angiogenesis inhibitor. An unexpected finding is that repeated cycles of antiangiogenic therapy are followed by prolonged tumour dormancy without further therapy.

In preliminary studies, drug resistance was not inducible in mice bearing Lewis lung carcinomas treated with TNP-470, a selective angiogenesis inhibitor which inhibits tumour growth⁴. Cancer patients treated with TNP-470 in clinical trials have, to date, not

developed resistance (unpublished observations). Furthermore, Kerbel proposed a hypothesis that antiangiogenic therapy could be a strategy to circumvent acquired drug resistance in cancer therapy⁵. Endostatin³, a 20K C-terminal fragment of collagen XVIII, is a specific inhibitor of endothelial cell proliferation, and has no obvious effect on resting endothelial cells, nor on a variety of normal, transformed or neoplastic cells³. In our report of the discovery of endostatin, we showed that systemic administration of endostatin to tumour-bearing mice resulted in regression of tumours to a microscopic size³. A dormant state, without evidence of toxicity, could be maintained for as long as endostatin was administered.

To demonstrate that antiangiogenic therapy does not induce drug resistance, we treated tumour-bearing mice with endostatin in a similar way in the present study. However, as soon as tumours had regressed, endostatin treatment was discontinued and the tumour was allowed to re-grow. Lewis lung carcinoma repeatedly regressed during six treatment cycles (Figs 1 and 2) extending over a period of 185 days (one mouse day is equivalent to 35 human days). Endostatin therapy was resumed when tumours had reached a mean volume of 350–400 mm³. Control mice were treated with saline and their tumours reached a mean volume of 10,005 ± 1,361 mm³ by 27 days. There was no evidence of drug resistance in the treated mice. At the end of the sixth cycle, the rate of tumour regression induced by endostatin was essentially the same as in the first cycle. Endostatin had a synchronizing effect upon the rate of tumour regression between animals, so that tumours arrived at the dormant state within 3–5 days of each other. Further synchronization was imposed by the experimental design, that is, treatment was continued until all tumours had fully regressed. After the sixth cycle of endostatin, all tumours remained dormant after finishing therapy as barely visible subcutaneous nodules varying in size from 5–50 mm³, throughout the next 159 days, when the experiment was terminated. This measurement includes skin thickness, so that the histological size of the dormant tumour is <2 mm³ (Figs 2 and 3).

In contrast, drug resistance developed rapidly when Lewis lung carcinomas of similar size were treated with cytotoxic chemotherapy. Lewis lung carcinoma is one of the most difficult tumours to treat by anti-cancer drugs in the screening panel of the National Cancer Institute⁶. Cyclophosphamide was chosen because it is the most effective single agent against Lewis lung carcinoma, yet few animals are cured⁷. At the maximum tolerated dose of 150 mg kg⁻¹ every other day for three doses, we found that Lewis lung carcinomas of between 100 and 500 mm³ remained stable for approximately 13 days and then began to re-grow (Fig. 4a). To allow for bone marrow recovery⁷, repeated cycles of cyclophosphamide were administered every 21 days to animals bearing Lewis lung carcinomas. Although survival was prolonged, acquired drug resistance developed after the second cycle and the mice died of rapidly progressing tumours despite continued chemotherapy (Fig. 4b).

T241 fibrosarcoma underwent repeated regression during four cycles of endostatin therapy over a period of 160 days. All tumours became dormant after therapy and failed to re-grow throughout the remaining 103 days of the experiment (Fig. 1b). To date, five mice have been killed but the remaining mice still have dormant tumours after therapy. Again, no drug resistance was evident. B16F10 melanoma regressed during two cycles over a period of 80 days, without evidence of drug resistance (Fig. 1c). Tumours in all mice remained dormant after the completion of two cycles of endostatin therapy through the end of the experiment 165 days later. All endostatin-treated mice in each tumour system remained healthy and gained weight normally.

A surprising finding was that for each tumour type there was a given time period of endostatin therapy following which the tumours did not resume growth after therapy was discontinued. Tumours remained dormant at a barely visible size while the animals were observed for 103 to 165 more days after the beginning of the dormant state (Fig. 1). At the end of this period mice in each group were killed for

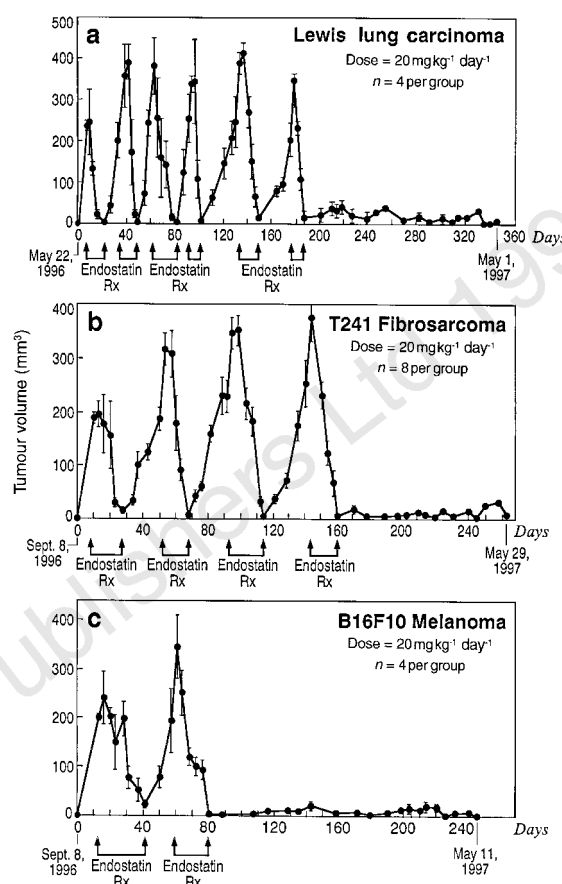


Figure 1 Cycled endostatin therapy of tumours grown in the subcutaneous dorsa of mice. **a**, Lewis lung carcinoma. When tumour volume reached 250 to 400 mm³, endostatin therapy was initiated. Tumours in four untreated animals grew to 10,005 ± 131 mm³ by day 27 (data not graphed). **b**, T241 fibrosarcoma. Endostatin therapy was initiated when the mean tumour volume was 200 mm³ and in subsequent cycles when the tumour volume was greater than 300 mm³. Tumours in four untreated animals grew to 9,127 ± 1,183 mm³ by day 28. **c**, B16F10 melanoma (a subclone of cells supplied by Isaiah Fidler, Houston). Endostatin therapy was initiated when the mean tumour volume was 230 mm³ for the initial cycle and at 350 mm³ for the second cycle. (Endostatin 20 mg per kg per day = 400 micrograms per 20 gram mouse.) Tumours in four untreated animals grew to 7,431 ± 1,122 mm³ by day 41.

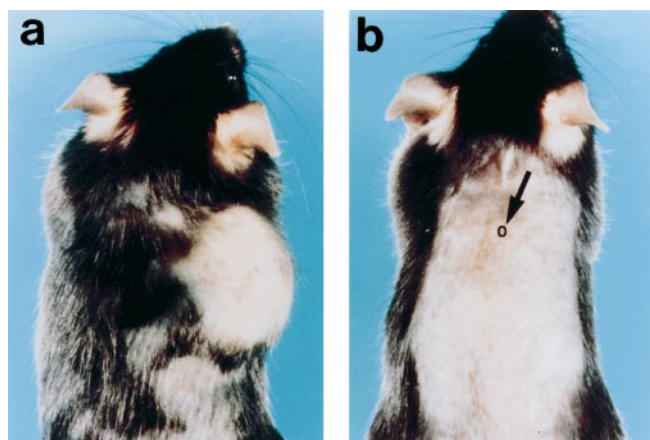


Figure 2 **a**, Lewis lung carcinoma at day 40, before endostatin therapy has been resumed in the second treatment cycle. **b**, Lewis lung carcinoma at day 52 after 12 days of endostatin therapy. The residual subcutaneous tumour is indicated by the arrow and circle.

histological study. Histological sections of the primary site revealed a residual tumour of microscopic size (Fig. 3). The endostatin-treated dormant tumours ranged in size from 0.06 to 2.23 mm³.

Histological sections revealed certain similarities among all three tumour types (Fig. 3). The tumours were hypovascular, as shown by one or more thin-walled vascular structures that resembled dilated capillaries containing thin quiescent endothelium, and had little or no active angiogenesis. Both proliferating and apoptotic tumour cells were present in a pattern similar to that previously seen with dormancy induced by angiogenesis blockade^{3,8}. All tumours were within the size limit of a tumour cell population that can survive without blood vessels, for example, pre-vascular *in situ* carcinomas, tumour spheroids in the vitreous⁹ or dormant lung micro-metastases^{8,10}. Some microscopic tumours were mildly infiltrated by leukocytes and others contained focal necrosis. Lungs from two mice in each group were examined for metastases. No lung metastases were observed grossly or in serial microscopic sections. This is of interest because untreated mice bearing primary Lewis lung carcinomas of 400 mm³ in size virtually all have microscopic lung metastases. Therefore, lung metastases were apparently eradicated by the cycling endostatin therapy.

To rule out the possibility that the observed dormancy was the result of an unprecedented type of immune response, animals with dormant tumours were inoculated with a tumour of the same type at a remote site on the left flank. Lewis lung carcinoma, inoculated on day 335, formed tumours which grew rapidly in all four mice, followed the same growth curve as the untreated controls, and reached a mean volume of 573 ± 74 mm³ by day 344. On day 203, T241 fibrosarcoma cells were injected subcutaneously into two mice at a remote site from the original tumour. Tumours grew rapidly and followed the growth curve of untreated controls and reached a mean volume of 1,100 mm³ 17 days later. Two more mice with dormant tumours received inoculations of T241 fibrosarcoma cells at a remote site on day 225. The tumours reached a mean volume of 2,000 mm³ 16 days later. Two mice received an inoculation of melanoma cells at a remote site at day 203. The tumours grew at a similar rate as untreated tumours and reached a mean volume of 550 mm³ by 17 days later. Melanoma cells were inoculated into the two remaining mice on day 225. The tumours grew to 2,600 mm³ over the next 20 days. As with the primary tumours, the reimplanted tumours were analysed histologically (Fig. 3a, d, g). The growth of Lewis lung carcinoma, fibrosarcoma and melanoma on a schedule similar to untreated control animals argues that the mice had not developed immunity to their original tumour implant.

These results provide, to our knowledge, the first formal evidence

that a specific angiogenesis inhibitor does not induce drug resistance in three different types of murine transplantable tumours. These results also show that repeated cycles of endostatin therapy induce tumour dormancy that persists indefinitely after therapy. While the mechanism of this unexpected finding is not yet clear, it illustrates the powerful control exerted by the vascular endothelial cell population over the tumour cell population^{11,12}. The lack of drug resistance after prolonged cyclic therapy by endostatin may be understood in terms of the relative diversity of the two predominant cell compartments in a tumour, the endothelial cell and tumour cell populations^{11,12}. Beyond this, the mechanism of this self-sustained dormancy in all three tumour types remains unknown. We have previously observed the onset of prolonged tumour dormancy after discontinuation of angiostatin therapy of human prostate carcinoma (PC-3) in immunodeficient SCID mice¹⁰. Of seven treated mice, four were killed for histological study, and residual tumour was found in only one mouse. Two of the three remaining mice lived for more than a year and died without evidence of recurrence. The third mouse died early of recurrent tumour. When we treated 300 mm³ Lewis lung carcinomas with angiostatin plus endostatin (both at 20 mg kg⁻¹ day⁻¹, *n* = 4) for 25 days, tumours regressed completely. All mice remained healthy and without detectable tumour for 11 months after therapy, at which time they were killed and all were tumour free (data not shown). Taken together, these results suggest that whatever is responsible for the onset of tumour dormancy after repeated cycles of endostatin therapy may be related to the total dose and time to which the tumour and its host endothelial cells are exposed to the angiogenesis inhibitor.

It remains to be seen whether lack of drug resistance will be a general property of angiogenesis inhibitors. On the basis of our results with endostatin, it may be predicted that those angiogenesis inhibitors that specifically or selectively target vascular endothelial cells will be less likely to induce resistance than inhibitors which target a tumour-derived mediator of angiogenesis. As an example of the latter, tumours which produce basic fibroblast growth factor (bFGF) as their predominant angiogenic mediator, may initially respond to interferon alpha-induced suppression of bFGF production by tumour cells¹³. However, as cells arise which produce other angiogenic factors, the tumour itself may acquire resistance to interferon alpha. Tumours could potentially become resistant to an angiogenesis inhibitor as specific as endostatin, should they generate a degrading enzyme, but to date we have not observed this. If in the future, antiangiogenic therapy can be used to help conventional anti-cancer therapies such as surgery, chemotherapy, radiotherapy or immunotherapy, angiogenesis inhibitors that do

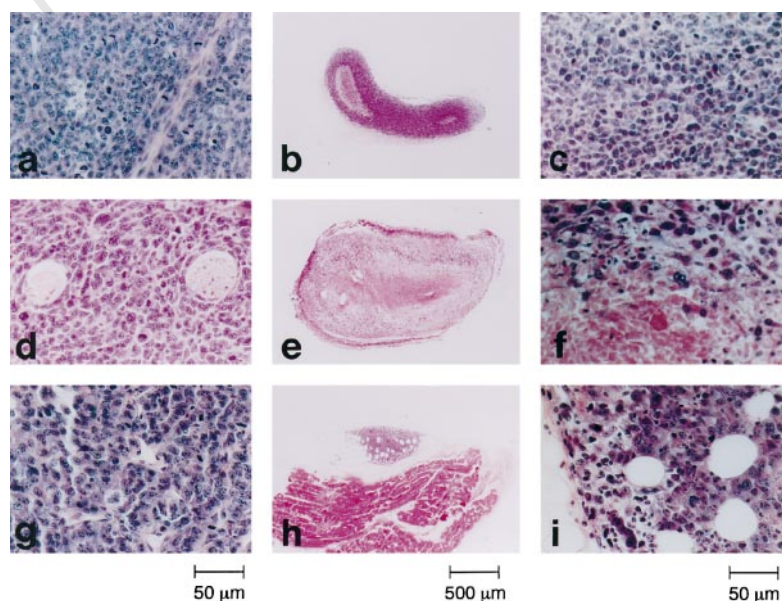


Figure 3 Representative histology of dormant tumours treated with cycled endostatin and of tumours implanted in the left flank after the original tumour became dormant. **a**, Lewis lung carcinoma (250×) 9 days after implantation in the left flank 152 days after the original tumour became dormant. **b**, Dormant endostatin-treated Lewis lung carcinoma 159 days after therapy (25×). The residual tumour measures 0.420 by 1.785 mm (Nikon Projectorscope) for a volume of 0.163 mm³. **c**, Higher power (250×) of the dormant Lewis lung carcinoma in **b**. **d**, T241 fibrosarcoma (250×) 16 days after implantation in the left flank 44 days after the original tumour became dormant following discontinuation of endostatin. **e**, Endostatin-treated T241 fibrosarcoma (25×) on day 263 after the tumour had been dormant for 103 days after therapy. The tumour measures 1.398 by 2.199 mm (volume = 2.23 mm³). **f**, Higher power (250×) of the dormant T241 fibrosarcoma in **e**. **g**, B16F10 melanoma (250×) 16 days after implantation in the left flank 124 days after the original tumour became dormant following discontinuation of endostatin. **h**, Endostatin-treated B16F10 melanoma (25×) on day 245 after the tumour had been dormant for 165 days after therapy. The residual tumour measures 0.427 mm by 0.652 mm (volume = 0.06 mm³). The large vacuoles are fat cells. **i**, Higher power (250×) of the dormant B16F10 melanoma in **h**.

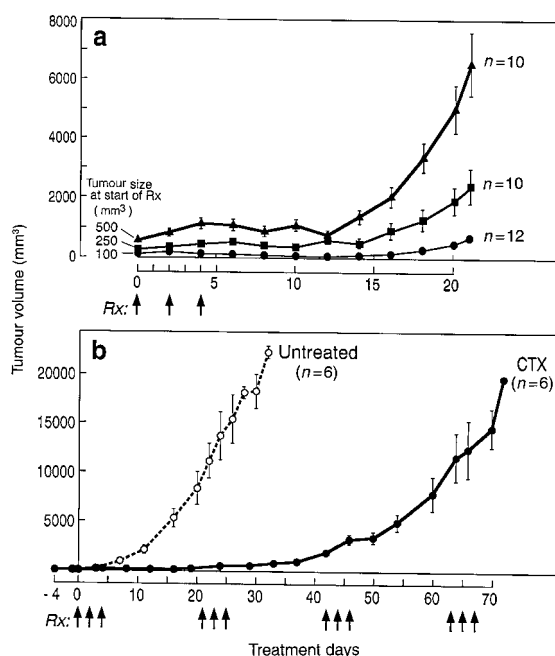


Figure 4 Treatment of Lewis lung carcinoma with cyclophosphamide. **a**, Cyclophosphamide therapy (150 mg kg^{-1}) was initiated when mean tumour volumes reached 100, 250 or 500 mm^3 and was repeated every other day for three doses (arrows) to a maximum tolerated total dose of 450 mg kg^{-1} . Tumour volumes are recorded as mean $\pm$ s.e.m. **b**, Cycled therapy with cyclophosphamide. Mice bearing carcinomas of 100 mm^3 were treated with the maximum tolerated dose of cyclophosphamide (CTX). The treatment cycle was repeated every 21 days (arrows), the shortest interval of recovery which these mice can tolerate without mortality. Comparable control mice received only saline (untreated). Tumour volumes are plotted as mean $\pm$ s.e.m.

not induce drug resistance may be valuable for long-term maintenance therapy. □

Methods

Production of recombinant endostatin. Recombinant murine endostatin was produced by *Escherichia coli* and purified as previously described³, with the following modification. The bacteria were pelleted and resuspended in 8 M urea, 10 mM β -mercaptoethanol and 10 mM Tris-HCl pH 8.0 containing 10 mM imidazole, and incubated for 1–2 h. In subsequent steps, β -mercaptoethanol was not used.

Mouse studies. Tumours were grown in the dorsal subcutaneous space of 6-week-old male C57BL/6 mice as previously described³. Tumours were measured in two dimensions by calipers at roughly every 4–6 days and volume was calculated as $\text{width}^2 \times \text{length} \times 0.52$. Recombinant mouse endostatin was delivered to mice as a suspension in PBS³. The precipitated endostatin was resuspended in PBS and stored at -20°C until use. The suspension was thawed to 4°C , vortexed and resuspended through a 26-gauge needle. The purified, but poorly soluble endostatin suspension was injected slowly with a 30-gauge needle into the subcutaneous dorsa of mice at a site remote from the inoculated tumours. The concentration of endostatin was adjusted with PBS so that each mouse received 0.2 ml per 20 g of body weight at a dose of $20 \text{ mg kg}^{-1} \text{ day}^{-1}$. Histological analysis of the tumours was performed as previously described³.

Cyclophosphamide therapy. To minimize gastrointestinal dysfunction and weight loss from chemotherapy, mice were pretreated with dexamethasone 1 mg kg^{-1} (Elkins-Sinn, New Jersey) and ondansetron 3 mg kg^{-1} (GlaxoWellcome, North Carolina) subcutaneously 30 min before administration of cyclophosphamide. Cyclophosphamide (Mead Johnson, New Jersey) was reconstituted with sterile water (25 mg ml^{-1}) and maintained as a stock solution at 4°C for up to 6 days (manufacturer's recommendation). The stock solution was diluted with sterile saline to 15 mg ml^{-1} just before dosing. Mice were weighed and received 0.01 ml g^{-1} body weight by intraperitoneal injection to achieve a dose of 150 mg kg^{-1} .

Received 24 July; accepted 9 September 1997.

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Acknowledgements. We thank R. Cotran for analysis of the histological sections, E. Flynn for preparation of the histological sections, K. Keough for help with protein purification, L. DeSantis for photography and S. Moscovitz of Advanced Medical Graphics for help with the figures. This study was supported by a grant to the Children's Hospital from Entremed (Rockville, Maryland) and by NIH grants. T.B. is a recipient of an Erwin-Schrödinger-Stipendium (Fond zur Förderung der Wissenschaftlichen Forschung, Austria). T. Browder is a recipient of an American Cancer Society Career Development Award.

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A plastid organelle as a drug target in apicomplexan parasites

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Parasites of the phylum Apicomplexa include many important human and veterinary pathogens such as *Plasmodium* (malaria), *Toxoplasma* (a leading opportunistic infection associated with AIDS and congenital neurological birth defects), and *Eimeria* (an economically significant disease of poultry and cattle)^{1–4}. Recent studies have identified an unusual organelle in these parasites^{5–7}: a plastid that appears to have been acquired by secondary endosymbiosis of a green alga⁷. Here we show that replication of the apicomplexan plastid (apicoplast) genome in *Toxoplasma gondii* tachyzoites can be specifically inhibited using ciprofloxacin, and that this inhibition blocks parasite replication. Moreover, parasite death occurs with peculiar kinetics that are identical to those observed after exposure to clindamycin and macrolide antibiotics^{8,9}, which have been proposed to target protein synthesis in the apicoplast^{9,10}. Conversely, clindamycin (and functionally related compounds) immediately inhibits plastid replication upon drug application—the earliest effect so far described for these antibiotics. Our results directly link apicoplast function with parasite survival, validating this intriguing organelle as an effective target for parasitocidal drug design.

Parasitic diseases are on the increase all over the world. The rapid emergence of drug resistance in malaria¹¹ and *Eimeria*¹², and the prevalence of *Toxoplasma*, *Cryptosporidium* and other diseases for which effective treatment is either unavailable or impracticable in immunocompromised patients¹³, lend renewed impetus to the search for new chemotherapeutic agents. The discovery of a novel organelle in apicomplexan parasites^{5–7} has received considerable attention^{14,15}, but the function of this apicomplexan plastid, or apicoplast, remains unknown. To investigate the role of this parasite-specific organelle and test whether it could be targeted by

Table 1 Plastid/nuclear genome ratio in control and drug-treated *T. gondii*

Drug treatment (concentration)	After 48 h drug treatment (1st infectious cycle)	After 72 h drug treatment (2nd infectious cycle)
None (control)	5.5 ± 1.1 [8]	NA
Ciprofloxacin (25 µM)	0.53 ± 0.33 [5]	0.23 ± 0.05 [4]
Clindamycin (1 µM)	2.2 ± 0.02 [5]	0.59 ± 0.12 [5]
Azithromycin (1.3 µM)	2.8	0.61
Chloramphenicol (20 µM)	4.0	2.3
Pyrimethamine (1 µM)	5.7 ± 1.6 [2]	NA
Tetracycline (100 µM)	5.2 ± 0.6 [2]	NA
Atovaquone (1 µM)	6.0 ± 0.7 [3]	NA
Anisomycin (100 nM)	5.4	NA

Ratios are mean ± s.d. from quantitative DNA hybridizations like that shown in Fig. 2; sample size (where >1) is indicated in brackets. NA, not applicable.

parasiticidal treatments, we used inhibitors of prokaryotic DNA gyrases to block replication of the 35-kilobase (kb) circular apicoplast genome⁵.

Ciprofloxacin and other fluoroquinolones are effective inhibitors of a wide range of prokaryotic gyrases^{16,17}. These drugs do not inhibit eukaryotic gyrases or mitochondrial DNA replication, but studies in plant systems indicate that plastid gyrases are sensitive to fluoroquinolones¹⁸. Although plastid- or prokaryotic-type gyrases have not yet been identified in any apicomplexan parasite, they must be present to permit replication of the circular apicoplast genome⁵. Treatment of intracellular *T. gondii* tachyzoites with ciprofloxacin reveals specific depletion of extranuclear DNA staining (Fig. 1). This extranuclear DNA, immediately apical to (but distinct from) the nucleus, co-localizes with apicoplast-specific DNA probes used in *in situ* hybridization⁷.

Quantitative hybridization analysis was used to examine copy number of the 35-kb apicoplast DNA genome, using probes specific for single-copy genes in either the nuclear or plastid genome. As shown in Fig. 2 and Table 1, the apicoplast genome is present at >5 copies per haploid nuclear genome in untreated parasites. Treatment with 25 µM ciprofloxacin, however, specifically reduced the plastid genome copy number by >10-fold over the course of replication within the infected cell. Copy number was further

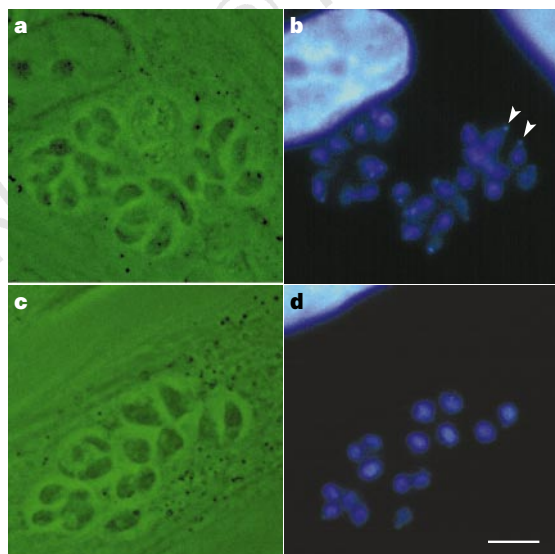


Figure 1 DAPI staining of intracellular parasites treated with ciprofloxacin reveals a loss of apicoplast DNA. Intracellular parasites grown in primary human fibroblasts³⁰ were fixed in formyl saline, stained with 4',6-diamidino-2-phenylindole (DAPI), and visualized using phase-contrast optics (**a**, **c**) or UV illumination (**b**, **d**). *T. gondii* tachyzoites divide synchronously within an intracellular parasitophorous vacuole⁹, oriented with their apical ends pointing outwards. Extranuclear DNA (arrowheads) corresponds to the apicomplexan plastid (apicoplast), not the mitochondrion⁷. Relative to untreated controls (**a**, **b**), staining of apicoplast DNA is reduced in parasites treated for >24 h with 25 µM ciprofloxacin (**c**, **d**). Scale bar, 10 µm.

reduced after parasites lysed out of the initial host cell and took up residence in a new fibroblast (second infectious cycle). Intracellular *T. gondii* tachyzoites divide about every 7 hours⁹, and this rate was unaffected by ciprofloxacin as long as parasites remained in the host cell where they were treated, which was typically for about 48 h (Fig. 3). The 10-fold reduction in relative copy number of the plastid genome therefore indicates that plastid genome replication is inhibited by ~50% following ciprofloxacin treatment.

This effect is not simply the result of more rapid degradation or instability of the plastid genome in treated parasites, as other compounds that kill *T. gondii* showed normal ratios of plastid-to-nuclear genomes for as long as intact DNA could be recovered from dying parasites (Table 1). Pyrimethamine inhibits nuclear DNA replication by starving the parasite of pyrimidines¹⁹, whereas tetracycline targets mitochondrial protein synthesis¹⁰, and atovaquone probably inhibits cytochromes in the mitochondrion^{20,21}; anisomycin is an inhibitor of cytoplasmic protein synthesis⁹. Incubating *T. gondii* tachyzoites without host cells for several days also kills parasites⁹, but nuclear and plastid genome copy numbers decline in parallel (data not shown).

Although ineffective against *T. gondii* *in vivo*²², ciprofloxacin is toxic to *T. gondii* parasites *in vitro* with a minimal inhibitory concentration of ~5 µM. As shown in Fig. 3, there was no significant decline in parasite replication rate for tachyzoites growing inside the parasitophorous vacuole within the first host cell (doubling times were 7.0 h; 6.5 h for controls), but division was slowed immediately upon exit from the vacuole and entry into a new host cell (doubling time ~21 h). These distinctive kinetics of parasite death were previously observed for only a very few classes of antibiotics: lincosamides, macrolides and chloramphenicol⁹, all of which are transpeptidation inhibitors in prokaryotic systems²³. Clindamycin, azithromycin and spiramycin have all been used successfully to treat toxoplasmosis^{24,25}, and although it is not known how they work in *T. gondii*, it has been argued that apicoplast ribosomes are the only plausible target for these and other drugs^{8-10,26,27}.

We examined apicoplast genome copy number in *T. gondii* tachyzoites treated with clindamycin, azithromycin or chloramphenicol, in either the first or second infectious cycle⁹ (Table 1). These drugs significantly reduced plastid genome copy number relative to nuclear DNA, albeit less effectively than ciprofloxacin, presumably because they act indirectly (perhaps inhibiting the synthesis of proteins necessary for ribosomal function or the import of DNA replication machinery into the apicoplast). The extent to which these compounds inhibit plastid replication (clindamycin >

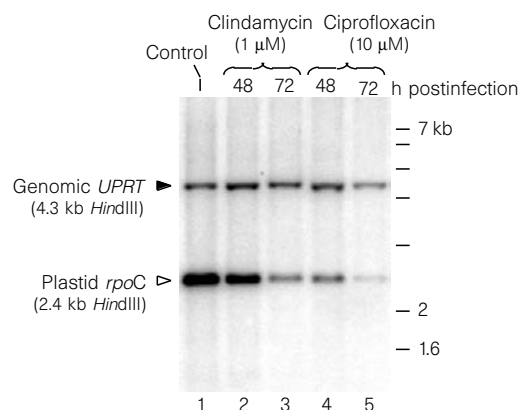


Figure 2 Quantitative Southern blots showing that ciprofloxacin and clindamycin selectively reduce plastid genome copy number relative to genomic nuclear DNA. Lane 1, untreated (control) parasites; lanes 2, 3, clindamycin-treated parasites; lanes 4, 5, ciprofloxacin-treated parasites. Parasites leave the first infected cell ~48 h post-infection⁹. 48-h samples are at the end of this first infectious cycle, and 60-h samples are in the second infectious cycle⁹ (Fig. 3). A quantitative analysis of these and related experiments is shown in Table 1.

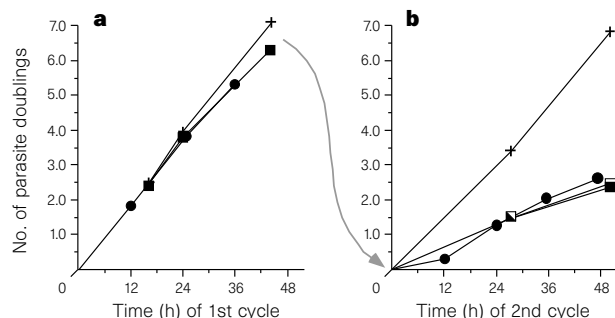


Figure 3 Ciprofloxacin and clindamycin inhibit intracellular tachyzoite division with the same peculiar kinetics. Parasites treated with either 25 µM ciprofloxacin (■, □) or 1 µM clindamycin (●) continued to divide with a doubling time virtually identical to untreated controls (+) throughout the first infectious cycle (a). Doubling times were reduced, however, immediately upon entry into the subsequent host cell (grey arrow), regardless of the continued presence or absence of drug (closed and open symbols, respectively) (b).

azithromycin > chloramphenicol) is qualitatively similar to their relative parasitocidal efficacy *in vitro*⁹. Even at very high doses, chloramphenicol is only weakly effective against *Toxoplasma* and long-term drug treatment is necessary²⁸, but the ratio of plastid-to-nuclear DNA continues to decline with prolonged treatment (data not shown). There is a reduction in copy number even in the first infectious cycle—the earliest result of treatment so far reported for these drugs. Long-term cultivation in clindamycin inhibited apicoplast DNA replication only slightly in clindamycin-resistant mutants²⁹ (plastid-to-nuclear ratios ranged from 2.7–3.7 for three independent mutants tested), but remained sensitive to ciprofloxacin and other drugs (not shown).

In combination, these results show that blocking replication of the 35-kb circular DNA that comprises the plastid genome in *Toxoplasma gondii* is lethal. Poisoning the apicoplast produces the same 'delayed-death' phenotype previously reported for clindamycin and mechanistically related drugs^{8,9}, implicating all of these drugs in apicoplast function. Loss of the apicoplast genome *per se* is not sufficient to inhibit *T. gondii* replication: parasites divide perfectly well inside the parasitophorous vacuole where they reside at the start of treatment, despite a reduction in plastid genome copy number to <1 per cell (compare 48 h of ciprofloxacin treatment in Fig. 3 and Table 1). Division is inhibited immediately upon entry into the second host cell, however, possibly as a result of improper function of the parasitophorous vacuole within the new host cell⁹. Although the metabolic function(s) of the apicoplast remains unknown, this organelle provides an effective target for parasitocidal chemotherapy. However, the peculiar kinetics of drugs acting on the apicoplast may complicate clinical application in cases where immediate efficacy is required. □

Methods

Quantitative DNA hybridization. RH-strain *T. gondii* parasites were cultivated with or without drug in primary human fibroblasts as described³⁰ and transferred to fresh host-cell cultures after each infectious cycle⁹. HindIII-digested total DNA from control or drug-treated parasites was separated by agarose gel electrophoresis and transferred to Nytran membranes according to standard procedures. Specific probes of identical size and specific activity were prepared for the nuclear and apicoplast genomes by random-primed ³²P-dATP-labelling of gel-purified PCR products to equal specific activity (1.1 × 10⁸ c.p.m. µg⁻¹). PCR primers 5'-TGCCTTTGCGGATGCGGAGTTCT-3' (sense) and 5'-CGGTACCGAAATCACCATTGC-3' (antisense) yield a 1,550-nucleotide (nt) product which specifically recognizes a 4.3-kb HindIII restriction fragment at the single-copy nuclear genomic *UPRT* locus (Genbank no. U10246); primers 5'-AGCTACTCCTATTGCCATAAATGGT-3' (sense) and 5'-TCGTTTCTTCTTAATAGTGGTGATAAAT-3' (antisense)

yield a 1,554-nt product which specifically recognizes a 2.4-kb HindIII band within the *rpoC* gene of the apicoplast genome (Genbank no. U87135). PCR conditions: ~100 ng total parasite DNA, 50 pmol each primer, 2.5 units *Taq* polymerase, 1.5 mM MgCl₂, 35 cycles of amplification (2 min at 94 °C, 2 min at 63 °C, 2 min at 72 °C).

Parasite-replication assays. Confluent cultures of HFF cells in 25-cm² T-flasks were inoculated with 10⁶ freshly lysed-out filter-purified parasite tachyzoites. After incubation at 37 °C for 8 h, the supernatant medium was aspirated to remove parasites that had not yet attached, replaced with fresh control or drug-containing medium, and the doubling of intracellular parasites was followed by counting the number of tachyzoites per parasitophorous vacuole at 12-h intervals (at least 50 vacuoles were scored at each time point)⁹. After parasite-mediated lysis of the infected HFF cell monolayer (~56 h postinfection), purified extracellular tachyzoites were inoculated into fresh cultures and followed for a further 48 h without drug. The number of parasite divisions since infection was determined during periods of log-phase growth as the log₂(parasite number)⁹.

Received 4 August; accepted 22 September 1997.

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Acknowledgements. Ciprofloxacin-HCl was provided by Bayer Corporation, Germany. We thank R. G. K. Donald for probes derived from the apicoplast and nuclear genomes and for developing the quantitative assays for plastid copy number, and members of our laboratory for discussion. This work was supported by research grants and a predoctoral training grant from the NIH. D.S.R. is a New Investigator of the Burroughs Wellcome Fund.

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Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling

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In *Drosophila*, members of the *frizzled* family of tissue-polarity genes encode proteins that are likely to function as cell-surface receptors of the type known as Wnt receptors, and to initiate signal transduction across the cell membrane^{1,2}, although how they do this is unclear. We show here that the rat protein Frizzled-2 causes an increase in the release of intracellular calcium which is enhanced by *Xwnt-5a*, a member of the Wnt family. This release of intracellular calcium is suppressed by an inhibitor of the enzyme inositol monophosphatase and hence of the phosphatidylinositol signalling pathway; this suppression can be rescued by injection of the compound *myo*-inositol, which overcomes the decrease in this intermediate caused by the inhibitor. Agents that inhibit specific G-protein subunits, pertussis toxin, GDP-β-S and α-transducin also inhibit the calcium release triggered by *Xwnt-5a* and rat Frizzled-2. Our results indicate that some Wnt proteins work through specific Frizzled homologues to stimulate the phosphatidylinositol signalling pathway via heterotrimeric G-protein subunits.

Stimulation of a G-protein-linked receptor or a tyrosine-kinase-linked receptor initiates the hydrolysis of a membrane-bound inositol lipid, generating at least two second messengers, diacylglycerol and inositol-1,4,5-trisphosphate (InsP₃). Diacylglycerol stimulates protein kinase C while InsP₃ promotes the release of intracellular calcium^{3,4}. As *Xwnt-5a* stimulates the release of intracellular calcium in zebrafish embryos⁵ and as the Frizzled family of candidate Wnt receptors is predicted to have a seven-span transmembrane topology similar to G-protein-coupled receptors^{6,7}, we investigated whether some Frizzled homologues could initiate Wnt signalling by activating the phosphatidylinositol pathway.

First we measured the intracellular calcium ion concentration (Ca²⁺) as an indicator of the activation of this pathway^{5,8} in embryos ectopically expressing rat Frizzled-1 (*Rfz-1*) and rat Frizzled-2 (*Rfz-2*)⁷. Zebrafish embryos were co-injected with RNA

encoding either *Rfz-1*, *Rfz-2* or globin, which had been mixed with Fura-2–dextran, a calcium-sensitive dye that is excluded from intracellular organelles. The embryos were then subjected to fluorescence ratio imaging throughout development to determine the spatial and temporal dynamics of cytoplasmic Ca²⁺ release^{5,8}. Expression of *Rfz-2* is sufficient to increase the frequency of Ca²⁺ release (Fig. 1a), whereas neither *Rfz-1* (Fig. 1b) nor a globin control (Fig. 1c) increased Ca²⁺ release above endogenous levels. *Rfz-2* injected embryos cultured in a calcium-free solution still show an increased frequency of Ca²⁺ transients (data not shown), indicating that the calcium is released from internal stores, which also occurs in response to *Xwnt-5a* (ref. 5). These data suggest that a member of the *frizzled* gene family stimulates a known second messenger and that *Rfz-1* and *Rfz-2* differ in their ability to couple to this pathway.

As *Rfz-2* increases the frequency of intracellular Ca²⁺ release like *Xwnt-5a* (ref. 5), these proteins could operate in a common pathway, in which case their co-expression should induce more-than-additive Ca²⁺ release. Using less RNA than for Fig. 1 to avoid saturating the embryonic Ca²⁺-transient responses, we found that both *Rfz-2* (Fig. 2a) and *Xwnt-5a* (Fig. 2b) induce intracellular Ca²⁺ transients above control levels (results not shown). When *Rfz-2* and *Xwnt-5a* were co-expressed, however, we observed a greater-than-additive increase in Ca²⁺-transient activity in terms of amplitude and distribution (Fig. 2c). In separate experiments using small amounts of *Rfz-2* and *Xwnt-5a* which by themselves do not induce intracellular Ca²⁺ release (data not shown), we found that *Xwnt-5a* (Fig. 2d) stimulated transients to a greater extent than *Xwnt-8* (Fig. 2e). We conclude that *Rfz-2* increases the frequency of intracellular Ca²⁺ transients in a way that is enhanced by *Xwnt-5a*. As zebrafish eggs contain endogenous Wnt proteins and Frizzled homologues (data not shown), the ability of ectopic *Rfz-2* and *Xwnt-5a* to stimulate Ca²⁺ transients when expressed individually may be due to their interaction with endogenous components of this signalling pathway.

To investigate whether this ability of *Rfz-2* to elevate [Ca²⁺]_i involves coupling to G proteins, we used pharmacological agents to target specific subclasses of G proteins. Pertussis toxin (PTX), which is specific for Gα_o and Gα_i subunits, prevents the catalysis of GDP–GTP exchange by the receptor, blocking both Gα- and Gβγ-mediated signalling⁹. The results in Fig. 3 show that PTX inhibits *Rfz-2* modulation of calcium flux, consistent with *Rfz-2* working through PTX-sensitive G proteins. Specifically, embryos were uniformly injected with Fura-2–dextran mixed with RNA encoding *Rfz-2* or *Xwnt-5a*, and a baseline of activity was established (data not shown). At the 64-cell stage, about 5 pg PTX was unilaterally co-injected with Texas red as a lineage marker. The subsequent time course of fluorescence imaging shows an inhibition in the frequency of transients on one side of the embryo uniformly expressing *Rfz-2* (Fig. 3a; *n* = 5) and the site of inhibition corresponds to the region of the embryo injected with PTX and Texas red (Fig. 3c). Similar

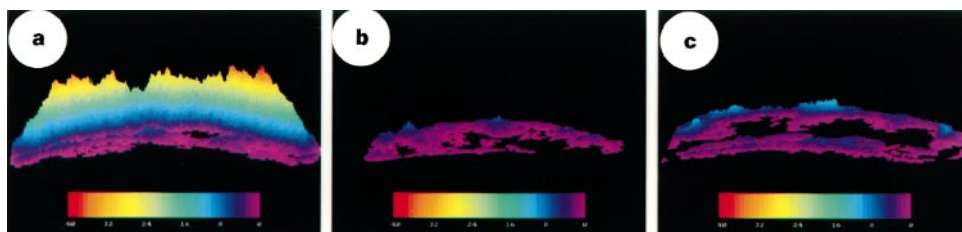


Figure 1 Differential calcium transient activity in zebrafish embryos induced by expression of *Rfz-2*, *Rfz-1* or globin RNA. Fura-2–dextran fluorescence ratio-image analysis composites representing the frequency of intracellular Ca²⁺ transients in embryos injected with **a**, *Rfz-2* RNA (24 pg); **b**, *Rfz-1* RNA (28 pg); or **c**, globin RNA (25 pg). Embryos injected with *Rfz-2* RNA have a much higher frequency of Ca²⁺ transients (6.83 ± 0.91 transients min⁻¹; *n* = 13) than embryos injected with *Rfz-1* RNA (3.03 ± 0.85 transients min⁻¹; *n* = 11). *Rfz-1*-induced

activity is not significantly different from a globin-injected control, whereas a Student's *t*-test yielded *P* < 0.01 between *Rfz-2* and *Rfz-1*. Data were collected during the time of mesoderm induction between the 32 and 64-cell stage and the 1,000-cell stage. The pseudocolour ratio images represented in this set are a compilation of a 40-min time course in which the colour bar represents the number of transients: red represents high numbers, violet represents lower numbers, and the peaks represent more active regions.

results were obtained with *Xwnt-5a* (data not shown). To control for nonspecific toxicity of PTX, we relied on the observation⁵ that ectopic *Xwnt-8* induces expression of a gastrula organizer gene, *gooseoid*. Control embryos were therefore injected with *Xwnt-8* at the 1-cell stage and with PTX and Texas red at the 32–64-cell stage. *In situ* hybridization to monitor *gooseoid* expression showed that *Xwnt-8* induced *gooseoid* expression to a similar extent in the absence or presence of PTX (data not shown). As a known activity of *Xwnt-8* was insensitive to PTX, we conclude that the inhibition by PTX of intracellular Ca^{2+} transients induced by *Rfz-2* and *Xwnt-5a* is specific.

To confirm this specificity of PTX, we tested whether the Ca^{2+} transients induced by *Rfz-2* could be blocked by the A-protomer of PTX, which lacks the lectin-like activity of the B-oligomer. Injection of Fura-2–dextran and *Rfz-2* RNA at the 1-cell stage, followed by injection of PTX A-protomer and Texas red into 1–2 cells at the 8–16-cell stage, inhibited Ca^{2+} transients (Fig. 3b; $n = 4$) in the region of the embryo injected with the A-protomer and lineage tracer (Fig. 3d).

To test further the involvement of G proteins in mediating the ability of *Rfz-2* and *Xwnt-5a* to induce Ca^{2+} transients, we used guanosine 5'-O-(3-thiodiphosphate), GDP- β -S, which is a non-hydrolysable GDP analogue that irreversibly inactivates G-protein-coupled events^{10,11}. *Rfz-2* or *Xwnt-5a* RNAs were injected with Fura-2–dextran into the 1-cell embryo to promote ubiquitous expression. After a baseline of activity was established and confirmed to extend throughout the embryo, a cocktail of GDP- β -S and Texas red was injected into a localized region at the 64-cell stage. Inhibition of *Rfz-2*-induced Ca^{2+} transients can be seen in the localized region (Fig. 3e), corresponding specifically to that region of the embryo injected with Texas red and GDP- β -S (Fig. 3g). The intracellular Ca^{2+} release triggered by both *Rfz-2* ($n = 6$) and *Xwnt-5a* ($n = 3$; data not shown) was inhibited at the site of GDP- β -S injection. We conclude that neither *Rfz-2* nor *Xwnt-5a* can increase the frequency of intracellular Ca^{2+} transients without G-protein function, which could involve either direct coupling of *Rfz-2* to a G protein or a G-protein requirement for increasing the release of intracellular Ca^{2+} .

Our results implicate G proteins as mediators of signalling

through *Rfz-2*, but do not distinguish whether this works through the $\text{G}\alpha$ or $\text{G}\beta\gamma$ subunits. The inhibitory G protein G_i activates the phosphatidylinositol pathway only via its $\beta\gamma$ subunits¹², so we tested whether induction of Ca^{2+} transients by *Rfz-2* operates through $\text{G}\beta\gamma$. We injected 1-cell embryos with *Rfz-2* RNA and Fura-2–dextran. At the 8–16-cell stage, 1–2 cells were then injected with Texas red and RNA encoding the α -subunit of transducin¹³, which traps $\beta\gamma$ subunits and thus prevents signalling¹⁴. In support of the hypothesis that *Rfz-2* induces Ca^{2+} transients through stimulation by G_i $\beta\gamma$ subunits of phosphatidylinositol signalling, α -transducin inhibits *Rfz-2*-mediated intracellular Ca^{2+} transients (Fig. 3f; $n = 4$) in that region of the embryo injected with α -transducin RNA and Texas red (Fig. 3h).

If *Rfz-2* and *Xwnt-5a* do induce intracellular Ca^{2+} release by activation of the phosphatidylinositol signalling pathway, then a specific inhibitor of this pathway should block the Ca^{2+} transients. The bisphosphonate compound L-690,330 is a competitive inhibitor of inositol monophosphatase (IMPase)¹⁵; inhibition of IMPase causes a decrease in the concentration of free inositol and in phosphatidylinositol cycling. We therefore tested the effect of L-690,330 on *Rfz-2*-induced intracellular Ca^{2+} release. Embryos were injected with *Rfz-2* and Fura-2–dextran at the one-cell stage, then one or several blastomeres were microinjected with L-690,330 and Texas red at the 8-cell stage, and embryos were subjected to image analysis. Figure 4a shows that L-690,330 inhibits Ca^{2+} transients induced by *Rfz-2*, and that the peak of inhibition correlates with the Texas red lineage tracer showing cells receiving the most L-690,330 (Fig. 4c). When application of the inhibitor is restricted to a small number of cells, the inhibition is also restricted to this small region of the embryo (data not shown; $n = 3$).

If this ability of L-690,330 to suppress intracellular Ca^{2+} release induced by *Rfz-2* results from inhibiting IMPase, then Ca^{2+} release should be rescued by providing the phosphatidylinositol cycle intermediate *myo*-inositol. We therefore injected embryos with uniformly distributed *Rfz-2* and Fura-2–dextran with L-690,330 mixed with *myo*-inositol and found that the release of intracellular Ca^{2+} was restored to levels observed in the absence of IMPase inhibitor ($n = 3$; data not shown). In another set of experiments,

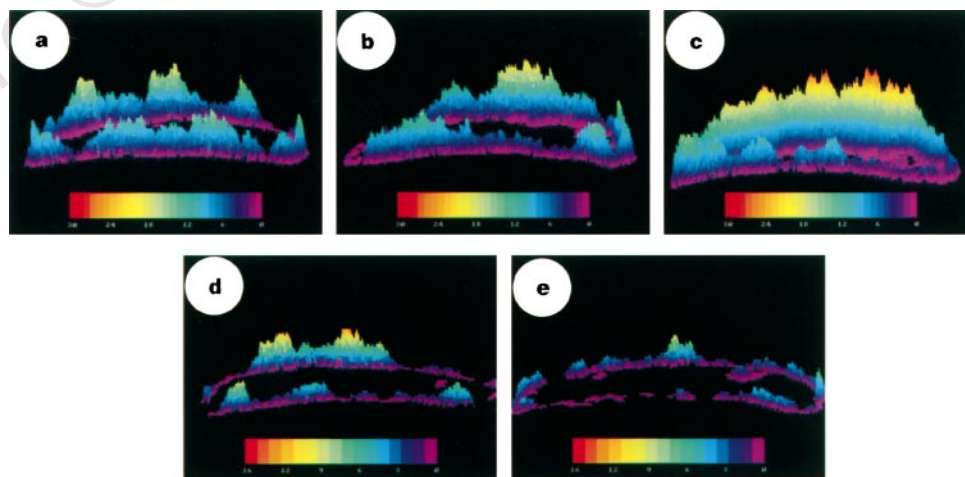


Figure 2 Induction of intracellular Ca^{2+} transients by *Rfz-2* in the presence of Wnt proteins. **a**, Calcium transient activity of an embryo expressing small amounts of *Rfz-2* RNA (10 pg); **b**, *Xwnt-5a* RNA (10–15 pg); and **c**, both *Xwnt-5a* (10–15 pg) and *Rfz-2* RNA (10 pg). At this dose of *Rfz-2* RNA (**a**), which is lower than that used for Fig. 1, the embryo has an average of 2.07 transients min^{-1} ; the embryo injected with *Xwnt-5a* RNA (**b**) has 1.50 transients min^{-1} . A representative embryo with the same low concentration and comparable fluorescence intensity of *Xwnt-5a* and *Rfz-2* together (**c**) has an average 6.49 transients min^{-1} . In **c**, *Rfz-2* was co-injected with the lineage marker Texas red (data not shown). The most intense regions of intracellular Ca^{2+} activity overlap with the Texas red-positive region in the right two-thirds of the embryo. **d**, Embryos uniformly expressing *Rfz-2* RNA (5–10 pg), at

a dose that does not stimulate a transient response, were then injected unilaterally at the 8-cell stage with *Xwnt-5a* RNA (6–10 pg). **e**, Embryos were injected as in **d** with *Rfz-2* RNA, then with *Xwnt-8* RNA (10 pg). When *Xwnt-5a* is injected to provide a putative ligand (**d**), there is an increase in intracellular Ca^{2+} in the region where RNA was distributed, as determined by the Texas red lineage marker (results with Texas red not shown). *Xwnt-8* had no synergistic effect with *Rfz-2* (**e**) even in the presence of higher levels of *Xwnt-8* RNA (30 pg) (data not shown). Owing to the reduced activity with lower doses of RNA, embryos were processed at higher contrast than for Fig. 1. Images in **a–c** are directly comparable to each other and are from one experiment; embryos in **d** and **e** are comparable and were obtained in a separate experiment.

embryos were injected at the 1-cell stage to aid uniform distribution of *Rfz-2* and Fura-2-dextran, followed by injection of L-690,330 and Texas red lineage tracer at the 8-cell stage (Texas red shown in Fig. 4e). Only a subset of cells at the 32-cell stage were then injected with *myo*-inositol and the fluorescent tracer Cy5 in order to test for

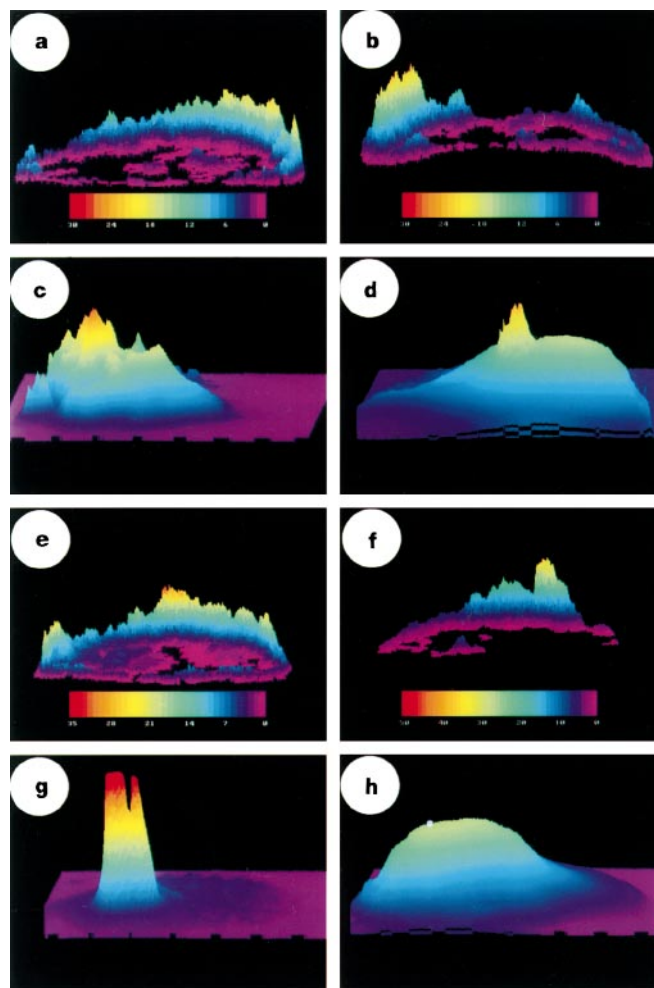


Figure 3 Pertussis toxin (PTX), the A-promoter of PTX, GDP- β -S, and α -transducin inhibit *Rfz-2*-induced release of intracellular Ca^{2+} . **a, c**, PTX inhibition of calcium transients. The profile of intracellular calcium release is shown in an embryo co-injected with *Rfz-2* RNA and Fura-2-dextran at the 1-cell stage (**a**). Calcium transients are evident on the right side of the embryo, and are repressed on the left side of the embryo. The repression on the left side correlates with the distribution of PTX, which was locally injected into one or more blastomeres at the 8–64-cell stage with Texas red as in **c** (a representative embryo injected at the 64-cell stage with PTX). **b, d**, A-promoter of PTX, the enzyme component of the holotoxin, inhibits calcium transients. *Rfz-2* RNA (20 pg) was injected with Fura-2-dextran into the 1-cell embryo. At the 8–16-cell stage, 1–2 pg of A-promoter mixed with Texas red was injected into 1–2 blastomeres. The transient profile (**b**) displays activity only in the left region of the embryo that did not receive the inhibitor or Texas red (**d**, representative embryo; $n = 4$). **e, g**, GDP- β -S inhibition of calcium transients. *Rfz-2* RNA (20 pg) was injected with Fura-2-dextran into the 1-cell embryo. After a baseline of activity was established and shown to be present throughout the embryo, 70–100 μl GDP- β -S (100 ng μl^{-1}) mixed with Texas red was injected into a localized region at the 64-cell stage. Calcium transients are seen on the right side of the embryo which did not receive GDP- β -S (**e**), and are repressed on the left side of the embryo, which did receive GDP- β -S, as evident from the localized Texas red (**g**). **f, h**, Expression of α -transducin inhibits calcium flux. *Rfz-2* RNA (20 pg) was co-injected at the 1-cell stage with Fura-2-dextran. At the 8–16-cell stage, α -transducin RNA (10–12 pg) was co-injected with Texas red into a localized region (**h**). *Rfz-2*-induced calcium transients are seen on the right of the embryo (**f**) in the region of the embryo that did not receive transducin RNA and Texas red (**h**, representative embryo; $n = 4$).

localized rescue of Ca^{2+} transients. We found that when *myo*-inositol application was restricted in this way, intracellular Ca^{2+} transients were rescued (peak in Fig. 4b) specifically in the region of the same embryo that expressed the highest amount of *myo*-inositol (Cy5 peak in Fig. 4d), confirming that the ability of *Rfz-2* to induce transients depends upon activation of the phosphatidylinositol cycle, linking this probable Wnt receptor to a known signal transduction pathway.

Our results define some of the early steps of signal transduction by *Rfz-2*, a member of the *frizzled* gene family of putative Wnt receptors, and by *Xwnt-5a*, a member of the *Wnt* gene family. They indicate that *Rfz-2* stimulates the phosphatidylinositol cycle through the $\beta\gamma$ subunits of pertussis-toxin-sensitive G proteins, leading to release of intracellular Ca^{2+} and diverse cellular responses. As $\text{G}\beta\gamma$ subunits also activate protein kinase C, which may be involved in Wnt signalling¹⁶, the responses by cells and embryos to signalling through Frizzled homologues could involve the stimulation of multiple cytoplasmic pathways. In vertebrate early embryos, regulation of the phosphatidylinositol pathway may be

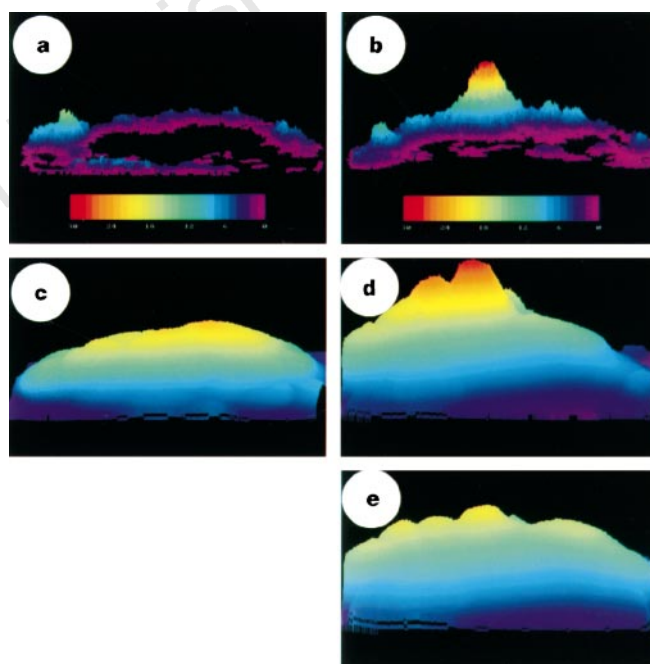


Figure 4 The IMPase inhibitor L-690,330 suppresses *Rfz-2*-induced intracellular Ca^{2+} release and is rescued by addition of *myo*-inositol. **a, c**, L-690,330 inhibits *Rfz-2* transients. *Rfz-2* RNA (20 pg) was injected into 1-cell embryos with Fura-2-dextran, resulting in broad distribution of RNA. Calcium transients are evident only near the ends of the embryo in **a** because L-690,330 and the Texas red tracer were injected into a subset of cells in the middle of the embryo at the 8-cell stage, as shown by the Texas red distribution (**c**). Image analysis was started within 10–25 min of injection of L-690,330. In the representative embryo in **a**, L-690,330 has a broad distribution, whereas other embryos with a more localized distribution of inhibitor show a restricted inhibition of calcium release, correlating with the area of maximal Texas red intensity ($n = 3$; data not shown). **b, d, e**, *myo*-inositol rescue of IMPase inhibition. Embryos were injected at the 1-cell stage with *Rfz-2* RNA and Fura-2-dextran, then with the inhibitor L-690,330 and the lineage tracer Texas red at the 8-cell stage. The Texas red signal gives a broad distribution throughout the embryo (**e**). At the 32-cell stage, *myo*-inositol was injected into only a few cells in the middle of the embryo, giving a localized distribution as shown by the lineage tracer Cy5 (**d**), and calcium transients were monitored within 10–25 min (1–2 cell cycles) of the last injection (**b**). The broad distribution of L-690,330 (**e**) inhibits *Rfz-2* intracellular Ca^{2+} release (**b**) except in the peak region of *myo*-inositol (**d**) ($n = 3$). Co-injection of L-690,330 with *myo*-inositol into embryos uniformly injected with *Rfz-2* RNA (20 pg) rescues the L-690,330 inhibition ($n = 3$; data not shown).

important for establishing the embryonic mesoderm and in other processes¹⁷. □

Methods

RNA injection and calcium imaging of zebrafish embryos. Rat Frizzled-1 and -2 (ref. 7) and the α -subunit of human transducin¹³ were subcloned into the vector pCS2⁺ to facilitate *in vitro* transcription of RNA and to add globin flanking sequences⁷. Zebrafish embryos were microinjected into one or more blastomeres with ~200 pl at the one-cell stage, and 50–100 pl at the 8–64-cell stages, with a cocktail of 5 mM Fura-2–dextran (Molecular Probes), 2.5 mM dextran-conjugated Texas red (Molecular Probes) or FluroLink Cy5 (Amersham), and RNAs as indicated. Fluorescence-ratio image analysis to determine the spatial and temporal dynamics of Ca²⁺ release has been described^{5,8}. Data were collected during the time of mesoderm induction between the 32 and 64-cell stage and the 1,000-cell stage. Quantitative data analyses were made using Microsoft Excel.

Synergy of the Rfz-2 receptor. To test for ligand specificity (Fig. 2), embryos were injected with enough Rfz-2 RNA (5–10 pg) and Fura-2–dextran not to stimulate a transient response with receptor alone. A pool of embryos giving equal Fura-2 fluorescence intensity and therefore having roughly equal amounts Rfz-2 RNA, were selected for a second set of microinjections. At the 8-cell stage, embryos were unilaterally injected with either *Xwnt-5a* (6–10 pg) RNA and Texas red or *Xwnt-8* (10 pg) and Texas red.

Pharmacological reagents. For Fig. 3, about 5 pg pertussis toxin (Sigma) or 1–2 pg pertussis toxin A-protomer (Calbiochem) was microinjected per embryo; GDP- β -S (7–10 pg; Calbiochem) was injected as the trilithium salt. To test whether inhibition of *Xwnt-5a*- and Rfz-2-induced intracellular Ca²⁺ release was influenced by the presence of traces of lithium in the injection cocktail, lithium was injected at concentrations 2- and 5-fold higher than that estimated to be present in the GDP- β -S compound: the lithium-control embryos did not display an inhibition like that seen with GDP- β -S. 50–100 pl of the IMPase inhibitor L-590,330 (Tocris Cookson) was injected per embryo from a 6–10 mM stock; 50–100 pl *myo*-inositol (Sigma) was injected from a 12.5 mM stock. L-690,330 injected from a 1 mM stock did not inhibit intracellular Ca²⁺ release in zebrafish embryos, but did at higher concentrations, when the inhibition lasted only 40–60 min after injection.

Received 29 July; accepted 29 August 1997.

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Acknowledgements. We thank W. B. Busa for advice on calcium imaging and for discussion; N. Nathanson for comments on the manuscript, and J. Hurley for transducin cDNA. This work was supported by the HHMI (R.T.M.) and grants from the NIH (V.G.C.); D.C.S. was supported by an NRSA fellowship from the NIH.

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Synergistic anti-apoptotic activity between Bcl-2 and SMN implicated in spinal muscular atrophy

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Spinal muscular atrophy (SMA) is a motor neuron disease characterized by degeneration of the anterior horn cells of the spinal cord. It is a common fatal autosomal recessive disorder and linkage studies have identified two candidate genes, SMN (ref. 1) and NAIP (ref. 2), both on chromosome 5q13. Although NAIP protein is known to have an anti-apoptotic function³, the function of SMN has been unclear and it shows no significant sequence similarity to any other protein. The SMN gene is deleted or interrupted on both chromosomes in nearly all SMA patients¹. Here we show that SMN interacts with Bcl-2, another anti-apoptotic protein⁴, and that co-expression of SMN with Bcl-2 confers a synergistic preventive effect against Bax-induced or Fas-mediated apoptosis, although SMN itself has only a weak anti-apoptotic activity. SMN^{Y272C}, which carries a missense mutation and was found in an SMA patient who exceptionally retained SMN on one allele¹, exerts no synergism with Bcl-2. Furthermore, the product of a truncated transcript lacking exon 7, which was derived from an SMN gene carrying an intragenic mutation or from the SMN copy gene 'BCD541' (ref. 1) retained in all SMA patients, had no synergistic activity but instead had a dominant-negative effect on full-length SMN. Our results indicate that an absent or decreased anti-apoptotic activity of SMN in concert with Bcl-2 underlies the pathogenesis of SMA.

During investigation of the anti-apoptotic function of Bcl-2, we searched for Bcl-2-interacting proteins using the yeast two-hybrid system. We screened a human thymocyte complementary DNA library and isolated three independent clones interacting with human Bcl-2 (hBcl-2). Two of these had almost identical fragments of the same gene, which encoded about 150 amino-acid residues with about 60% identity to the mouse Bad protein⁵, and probably represented the human *bad* gene. The third clone contained the entire coding sequence of human SMN, the gene mutated in spinal muscular atrophy¹.

The Bcl-2 family includes death agonists such as Bax, Bak, Bik, Bcl-x_s and Bid, as well as the death antagonists Bcl-2, Bcl-x_L and Bcl-w (ref. 4). Analysis in the yeast two-hybrid system revealed that SMN bound to hBcl-2, but not to human Bcl-x_L (hBcl-x_L), human Bax (amino acids 30–192) (hBax) or chicken Bcl-2 (cBcl-2); SMN also bound to SMN itself, as reported previously⁶ (Table 1).

To examine the interaction of SMN with Bcl-2 in mammalian cells, SMN fused at its amino terminus with the haemagglutinin epitope (HA-SMN) was expressed together with hBcl-2 by transient co-transfection of COS-7 cells, and the immunoprecipitates from lysates of these cells were western blotted. When both HA-SMN and hBcl-2 were expressed, an anti-HA-tag monoclonal antibody (12CA5) (data not shown) and an anti-SMN polyclonal antibody immunoprecipitated hBcl-2 (Fig. 1a). In addition, an anti-hBcl-2 antibody immunoprecipitated HA-SMN (Fig. 1b). We conclude that SMN and hBcl-2 proteins interact with each other in mammalian cells. We also observed that neither cBcl-2 nor mouse Bax (mBax)

coimmunoprecipitated with HA-SMN (data not shown), consistent with our results from the yeast two-hybrid system.

Subcellular fractionation localized SMN protein mainly to the heavy membrane fraction, with small amounts in the nuclear and light membrane fractions (Fig. 2a). Bcl-2 is localized similarly (Fig. 2b), as reported previously^{7,8}. Treatment with 0.5% Nonidet P40 detergent, which strips off the nuclear envelope, eliminated virtually all Bcl-2 protein from the crude nuclear fraction, but not SMN protein (N⁺ in Fig. 2a, b). These results indicate that SMN codistributes with Bcl-2 to several membrane compartments, but SMN also resides in the nucleoplasm⁶, and/or the inner nuclear membrane with which Bcl-2 is not associated. Immunocytochemistry also revealed a similar subcellular distribution of SMN and Bcl-2 (Fig. 2c, d).

In view of the pathology of SMA and the interaction between SMN and hBcl-2, we investigated whether SMN could enhance the anti-apoptotic activity of Bcl-2. To test the effects of SMN on Bcl-2 activity, we microinjected expression plasmids into HeLa cell nuclei, which allowed us to control the expression of gene products in cells with an identical genetic background. We first examined the effects of HA-SMN, hBcl-2 and cBcl-2 on Bax-induced apoptosis. Bax is a pro-apoptotic member of the Bcl-2 family and is required for the death of sympathetic neurons and motor neurons after trophic factor deprivation⁹, suggesting that Bax might be involved in neuronal death in motor neuron disease. About 80% of HeLa cells microinjected with 20 ng μL^{-1} of *bax* DNA underwent apoptosis within 3 h (Fig. 3a). When a high dose of HA-SMN or of human or chicken *bcl-2* expression plasmid was microinjected with *bax*, hBcl-

2 and cBcl-2 prevented apoptosis, whereas SMN did so only weakly (Fig. 4a), although SMN was shown to be expressed from injected DNA by immunocytochemistry (Fig. 2d). When SMN and human *bcl-2* DNAs were co-injected at high doses, the effect of SMN on Bcl-2 activity was barely detectable (data not shown). To investigate the possible synergy between SMN and Bcl-2, the concentrations of SMN and of human and chicken *bcl-2* DNAs were limited to the maximum that did not cause significant anti-cell death on their

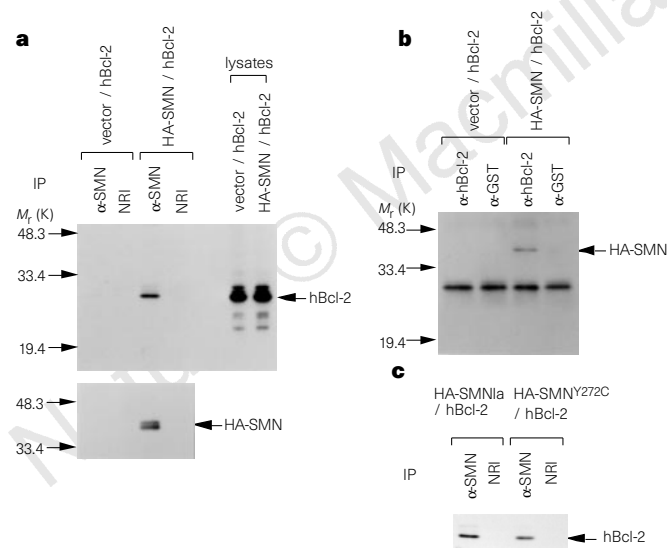


Figure 1 Co-immunoprecipitation of human Bcl-2 and SMN. **a**, Top, plasmids pCAGGS and pCAGGS-hbcl-2 (vector/hBcl-2) or pCAGGS-HA-SMN and pCAGGS-hbcl-2 (HA-SMN/hBcl-2) were co-transfected into COS-7 cells and cell lysates were immunoprecipitated (IP) using anti-SMN Ab (α-SMN) and normal rabbit IgG (NRI) as control antibody. Each sample was subjected to 12.5% SDS-PAGE and western blot analysis. Part of each precleared lysate obtained before immunoprecipitation was also loaded to show equivalence with Bcl-2 in lysates. hBcl-2 was detected using anti-hBcl-2 mAb 100. Bottom, specific immunoprecipitation of HA-SMN with anti-SMN antibody was confirmed by reprobing the membrane with anti-HA mAb 12CA5. **b**, Cell lysates were immunoprecipitated using rabbit anti-hBcl-2 Ab (α-hBcl-2) and anti-GST Ab (α-GST) was used as a control. After gel electrophoresis, HA-SMN was detected by anti-HA mAb 12CA5. A signal at ~30K on western blots is due to the rabbit IgG light chain detected by secondary anti-mouse IgG. **c**, Plasmids pCAGGS-HA-SMN1a or pCAGGS-HA-SMN^{Y272C} were co-transfected with pCAGGS-hbcl-2 (HA-SMN1a/hBcl-2 or HA-SMN^{Y272C}/hBcl-2, respectively) into COS-7 cells and cell lysates were immunoprecipitated using anti-SMN and normal rabbit IgG (NRI) as the control antibody. After gel electrophoresis, hBcl-2 was detected using anti-hBcl-2 mAb 100.

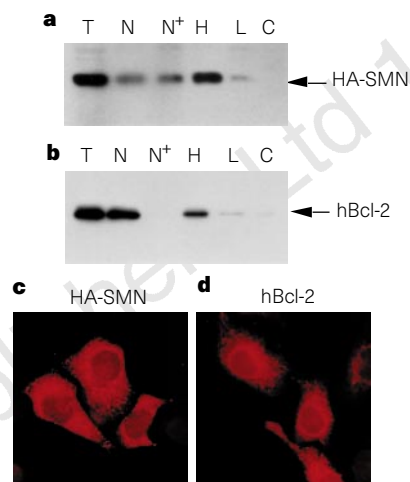


Figure 2 Intracellular localization of SMN protein. **a**, **b**, The subcellular localization of SMN and Bcl-2 was determined by a cell fractionation procedure⁸. Fractions of HeLa cells stably transfected with pCAGGS-hbcl-2 and pCAGGS-HA-SMN were standardized to represent equal numbers of cells in each fraction, and analysed by PAGE and western blotting with anti-HA mAb 12CA5 (**a**) and anti-hBcl-2 mAb 100 (**b**). T, total cell lysate; N, nuclear fraction; N⁺, nuclear fraction after treatment with 0.5% NP40; H, heavy membrane fraction; L, light membrane fraction; C, cytoplasmic fraction. Virtually identical results were obtained with stable transfectants expressing either Bcl-2 or SMN. **c**, **d**, HeLa cells were seeded on glass coverslips in medium, and 20 ng μL^{-1} HA-SMN DNA (**c**) or 2 ng μL^{-1} bcl-2 DNA (**d**) were microinjected. Two hours later, cells were fixed and immunostained using anti-HA mAb (**c**) or anti-human Bcl-2 mAb (**d**), with RITC-labelled second antibodies. Cells were visualized in a fluorescence microscope.

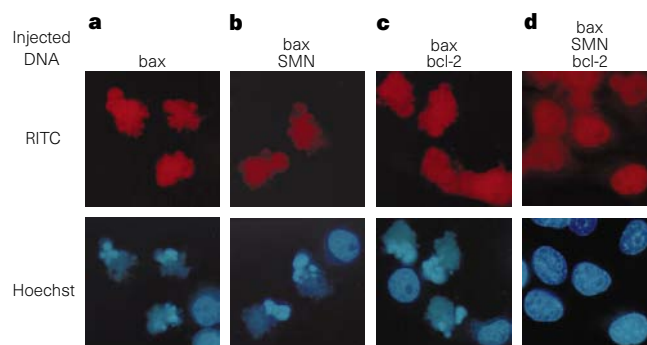


Figure 3 Fluorescence microscope analysis of synergistic prevention of Bax-induced apoptosis by SMN and Bcl-2. HeLa cells grown on coverslips were microinjected with the indicated DNAs in various combinations, together with RITC-conjugated BSA at 5.8 mg mL^{-1} . **a**, pCAGGS-mbax (bax); **b**, pAGGS-mbax and pCAGGS-HA-SMN (bax/SMN); **c**, pCAGGS-mbax and pAGGS-hbcl-2 (bax/bcl-2); **d**, pCAGGS-mbax, pCAGGS-HA-SMN and pCAGGS-hbcl-2 (bax/SMN/bcl-2). The concentrations of microinjected DNAs were as follows: pCAGGS-mbax, 20 ng μL^{-1} ; pCAGGS-HA-SMN, 20 ng μL^{-1} ; pCAGGS-hbcl-2, 2 ng μL^{-1} . Total DNA concentrations were adjusted to 200 ng μL^{-1} with DNA from the pCAGGS vector. After incubation for 3 h, cells were fixed, stained with Hoechst 33342, and examined by fluorescence microscopy for RITC (upper panels) and for Hoechst 33342 (lower panels). Photomicrographs in the upper and lower panels were taken from the same fields. Scale bar, 10 μm .

own. Although microinjection of $20 \text{ ng } \mu\text{l}^{-1}$ SMN DNA or $2 \text{ ng } \mu\text{l}^{-1}$ human *bcl-2* DNA had no significant effect on Bax-induced apoptosis (Figs 3b, c and 4b), co-injection of both DNAs at these concentrations had a synergistic preventive effect (Figs 3d, 4b), without affecting the amounts of each protein in injected cells (data not shown). This increased anti-apoptotic activity was unlikely to be due to overexpression of nonspecific proteins, because substitution of human *bcl-2* or SMN DNA by β_2 -microglobulin DNA had no effect (Fig. 4b). In NT2 cells, a human neuronal-precursor cell line, a similar synergistic effect of SMN and hBcl-2 was found (Fig. 4c), indicating that this synergistic function is not unique to HeLa cells.

To confirm that the enhancement of anti-apoptotic activity by co-expression of *bcl-2* and SMN DNAs was not an additive effect, we analysed the effect of SMN in combination with cBcl-2, which prevented apoptosis of HeLa cells as efficiently as did hBcl-2 (Fig. 4a) but which did not interact with SMN (Table 1). Unlike SMN and hBcl-2, co-injection of SMN with chicken *bcl-2* did not increase anti-apoptotic activity (Fig. 4b), indicating that the stimulation of anti-apoptotic activity by SMN co-injected with human *bcl-2* DNA was not merely additive but was due to the synergism.

We tested whether this synergistic effect of SMN and hBcl-2 was operative in another apoptosis-inducing system, by microinjecting HeLa cells with SMN DNA and *bcl-2* DNA at various concentra-

tions, incubating for 2 h to ensure adequate production of these proteins (data not shown), then treating with an agonistic anti-Fas antibody for 6 h; we found that $\sim 70\%$ of control vector-injected cells underwent apoptosis (data not shown). Like Bax-induced apoptosis, co-injection of SMN DNA with human *bcl-2* DNA drastically inhibited Fas-mediated apoptosis (Fig. 4d), whereas there was no synergistic effect when SMN DNA was co-injected with chicken *bcl-2* DNA (Fig. 4d), indicating that SMN and hBcl-2 act together to prevent Fas-mediated apoptosis.

Although the SMN gene is either deleted or interrupted on both chromosomes in most SMA patients, some retain the gene on an allele that carries a mutation such as a missense mutation (Y272C)¹, a frameshift deletion resulting in a premature stop codon¹⁰, or small deletions in introns near splice sites that probably cause disruption of splicing¹. As shown in Fig. 4e, the mutant SMN (SMN^{Y272C}) had no synergistic effect with human Bcl-2 against Bax-induced apoptosis, even when the concentration of the mutant SMN DNA was varied from 2 to $200 \text{ ng } \mu\text{l}^{-1}$. Immunofluorescence analysis revealed that SMN^{Y272C} expression was comparable with that of SMN when the same amounts of DNAs were injected (data not shown), indicating that SMN^{Y272C} was a loss-of-function mutant. Although SMN^{Y272C} binds to Bcl-2 and SMN (Table 1 and Fig. 1c), this binding appears to be insufficient for synergistic anti-apoptotic function.

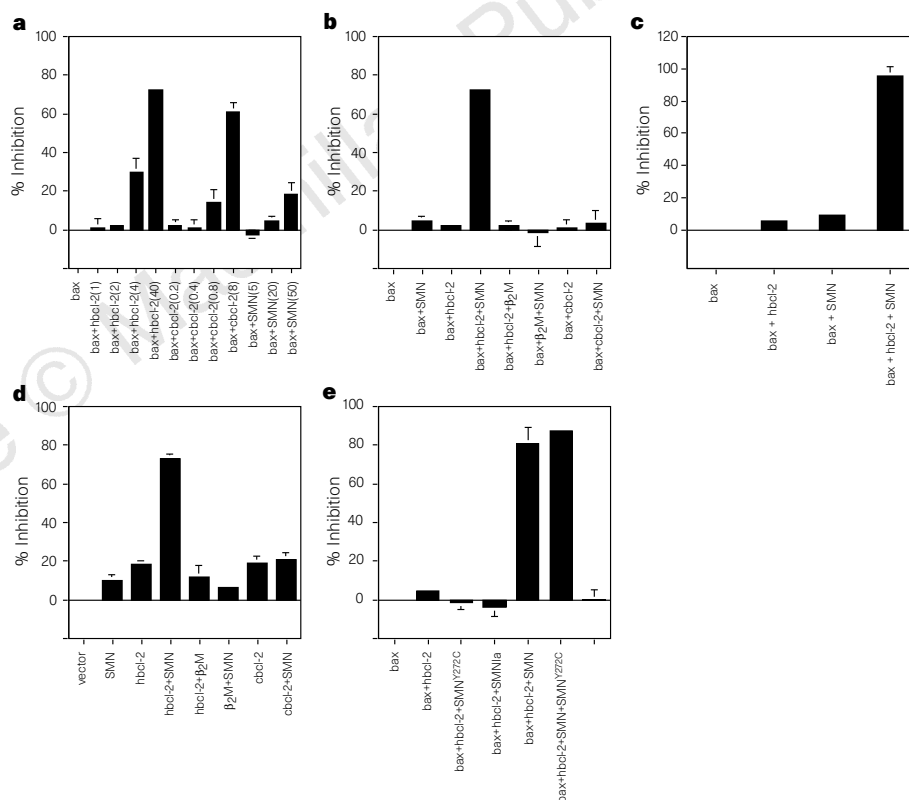


Figure 4 **a**, Per cent inhibition of Bax-induced apoptosis by hBcl-2, cBcl-2 and SMN. HeLa cells were microinjected with the indicated DNAs and apoptotic cells were counted after incubation for 3 h. About 80% of *bax*-injected cells underwent apoptosis. Per cent inhibition was calculated as follows: (% apoptosis in *bax*-injected cells) – (% apoptosis in the indicated DNA-injected rolls)/(% apoptosis in *bax*-injected cells), where % apoptosis is the percentage of apoptotic cells relative to injected cells. The concentration of *bax* DNA was $20 \text{ ng } \mu\text{l}^{-1}$ and concentrations of other DNAs are shown in parentheses ($\text{ng } \mu\text{l}^{-1}$). **b**, Synergistic inhibition of Bax-induced apoptosis by SMN and hBcl-2 in HeLa cells, assayed as for **a**. Concentrations of injected DNAs were as follows: *bax*, $20 \text{ ng } \mu\text{l}^{-1}$; SMN, $20 \text{ ng } \mu\text{l}^{-1}$; *hbc1-2*, $2 \text{ ng } \mu\text{l}^{-1}$; *cbcl-2*, $0.4 \text{ ng } \mu\text{l}^{-1}$. β_2 -Microglobulin (β_2 M) DNA was used as a control at $20 \text{ ng } \mu\text{l}^{-1}$ in (*hbc1-2* + β_2 M) and $2 \text{ ng } \mu\text{l}^{-1}$ in (β_2 M + SMN). **c**, Synergistic inhibition of Bax-induced apoptosis by SMN and hBcl-2 in NT2 cells. NT2 cells were microinjected with the indicated DNAs, and analysed after

incubation for 4 h. About 60% of *bax*-injected cells underwent apoptosis. Injected DNA concentrations ($\text{ng } \mu\text{l}^{-1}$): *bax*, 40; SMN, 20; *hbc1-2*, 2. **d**, Synergistic prevention of Fas-mediated apoptosis by SMN and hBcl-2. HeLa cells injected with indicated DNAs were incubated for 2 h and treated with an agonistic anti-Fas antibody ($1 \mu\text{g ml}^{-1}$) for 6 h. About 70% of vector-injected cells underwent apoptosis by this treatment. Injected DNA concentrations ($\text{ng } \mu\text{l}^{-1}$): SMN, 0.5; *hbc1-2*, 0.1; *cbcl-2*, 0.1; β_2 M, 0.5 in (*hbc1-2* + β_2 M) and 0.1 in (β_2 M + SMN). **e**, No synergism between SMN^{Y272C} or SMNla with hBcl-2; SMNla has a dominant-negative effect on inhibition of Bax-induced apoptosis by SMN. Injected DNA concentrations ($\text{ng } \mu\text{l}^{-1}$): *bax*, 20; SMN, 20; *hbc1-2*, 2; SMN^{Y272C}, 200; SMNla, 50. **a–e**, Total DNA was adjusted to $200 \text{ ng } \mu\text{l}^{-1}$ with DNA from pCAGGS vectors, except for SMN^{Y272C}, 40–90 cells were injected with each DNA combination. Results are expressed as the mean $\pm$ s.e.m. of three independent experiments.

Table 1 Yeast two-hybrid analysis

Plasmids	β -Galactosidase activity
pBTM116-hBcl-2 + pGAD10-SMN	++++
pBTM116 + pGAD10-SMN	—
pBTM116-RAD51 + pGAD10-SMN	—
pBTM116-hBcl-x _L + pGAD10-SMN	—
pBTM116-hBcl-x _L + pGAD10-hBax	++++
pBTM116-cBcl-2 + pGAD10-SMN	—
pBTM116-cBcl-2 + pGAD10-hBax	++++
pBTM116-SMN + pGAD10-hBax	—
pBTM116-hBcl-2 + pGAD10-hBax	+
pBTM116-SMN + pGAD10-SMN	++++
pBTM116-hBcl-2 + pGAD10-SMN ^{Y272C}	++++
pBTM116-RAD51 + pGAD10-SMN ^{Y272C}	—
pBTM116-SMN + pGAD10-SMN ^{Y272C}	++++
pBTM116-hBcl-2 + pGAD10-SMN ^Δ	++++
pBTM116-RAD51 + pGAD10-SMN ^Δ	—

The L40 reporter strain of yeast was co-transfected with 2 μ g of each of the indicated plasmids. Individual transformants were plated in SD/-Trp/-Leu/-His medium and grown for 4 d. Then colonies more than 1 mm in diameter were picked and streaked onto fresh SD/-Trp/-Leu/-His plates. Three days later, β -galactosidase activity was assayed. The human *bad* gene construct isolated in this study was used as a positive control for hBcl-x_L and cBcl-2; hBcl-2 was also used for hBax. RAD51 was a negative control. Symbols represent the time required for development of a strong blue colour: + + + +, less than 1 h; +, 3–4 h; —, more than 8 h, or never grown on SD/-Trp/-Leu/-His plates. SMN^Δ interacts with SMN, as assessed by yeast two-hybrid analysis⁶.

A genomic region of ~500 kilobases (kb) that carries the *SMN* gene is duplicated as an inverted repeat so that a copy gene of *SMN*, termed *BCD541* (ref. 1), is present. The *BCD541* gene has five silent base substitutions, but retains a protein-coding capacity identical to that of the *SMN* gene¹. The *BCD541* gene produces a truncated transcript (SMN^Δ mRNA) lacking exon 7, as a result of alternative splicing, in addition to a full-length transcript¹, whereas *SMN* generates only full-length mRNA¹. The *BCD541* gene is retained in all SMA patients¹, but the ratio of the amount of SMN^Δ mRNA to full-length mRNA is much larger in these patients than in normal individuals^{1,11}. SMN^Δ mRNA is also produced by a version of the *SMN* gene with a small deletion in intron 6 (ref. 1), as found in one patient. As shown in Fig. 4e, SMN^Δ had no synergistic effect with Bcl-2 but rather had a dominant-negative effect on SMN, explaining why the function of SMN is markedly decreased in SMA patients. Levels of either Bcl-2 or SMN expressed from injected DNA were not affected by co-expressed SMN^Δ (data not shown). As SMN^Δ binds Bcl-2 and SMN (Table 1, Fig. 1c and ref. 6), it might prevent interaction between SMN and Bcl-2, and thus exert a dominant-negative effect. SMN^{Y272C} did not exert any such dominant-negative effect (Fig. 4e), despite its binding to Bcl-2 and SMN, so the effect might depend on the mode of interaction.

Human Bcl-2 binds to and acts with SMN to prevent apoptosis, but chicken Bcl-2 does not. As human and chicken Bcl-2 have similar primary structures except in the loop region^{12,13}, which exerts a negative effect on Bcl-2 activity¹⁴, this loop structure might participate in the synergism of SMN and Bcl-2. Human Bcl-2 without the loop region (amino acids 31 to 92 replaced by four alanine residues) can still bind SMN, although less efficiently in yeast two-hybrid analysis (data not shown), so SMN presumably does not interact just with the loop region of Bcl-2, although the loop may affect the binding affinity. Further investigation is needed to understand the mechanism of this synergy.

NAIP is another candidate gene for SMA that also has an anti-apoptotic function; defects in this gene appear to be closely correlated with disease severity¹⁵. We have found that mutations in SMN in SMA patients¹ destroy its anti-apoptotic synergy with Bcl-2, suggesting that it is this defect that underlies the pathogenesis of SMA. The loss of function of both SMN and NAIP proteins is probably responsible for neuronal cell death in SMA, suggesting that anti-apoptotic proteins may regulate neuronal survival.

bcl-2-deficient mice show substantial degeneration of peripheral sensory and sympathetic neurons as well as of motor neurons¹⁶, but neuronal degeneration in SMA patients appears to be restricted to lower motor neurons. The survival of sympathetic and sensory neurons might be accounted for by the high expression of Bcl-2 in

these neurons which is maintained throughout life¹⁷ and might be sufficient to exert anti-apoptotic activity without SMN, in agreement with our microinjection experiments. Thus, SMN may be crucial only in cells such as motor neurons with decreased Bcl-2 expression. Our results provide some insight into the pathogenesis of neurodegenerative diseases and will help in the design of therapeutic strategies for these devastating disorders. □

Methods

Plasmids. The following plasmids were used in the yeast two-hybrid system: pBTM116-hbcl-2 for production of hBcl-2 lacking the C-terminal 13 amino acid residues fused to the DNA-binding domain of LexA; pBTM116-hbcl-x_L for production of a LexA-hBcl-x_L fusion; pBTM116-cbcl-2 for production of a LexA-cBcl-2 fusion; pGAD10-hbax for producing hBax lacking the N-terminal 29 residues fused to the Gal4-activating domain; and pBTM116-RAD51 as a control plasmid. pUC-CAGGS expression vector bearing a β -actin promoter and CMV enhancer, or pBC140 expression vector bearing a CMV promoter and enhancer, was used in mammalian cells. pCAGGS-BMG bearing mouse β_2 -microglobulin in pUC-CAGGS was also used as a control in microinjection experiments. The expression plasmid for HA-SMN was constructed so that the 9 codons of the HA epitope were fused to the second codon of SMN using PCR primers. SMN^{Y272C} DNA and SMN^Δ DNA were generated by PCR-based mutagenesis. The HA epitope was also fused to SMN^{Y272C} and SMN^Δ for immunoprecipitation and immunocytochemistry.

Yeast two-hybrid screening. The yeast strain L40 (*MATa trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*) was used. L40 harbouring pBTM116-hbcl-2 was transformed with 100 μ g of an oligo(dT) and/or random-primed human thymocyte cDNA fusion library in a Gal4-activating domain vector (pGAD10, Clontech) using lithium acetate. Transformants were selected by culture on SD/-Trp/-Leu/-His plates for 4 days and streaked out onto fresh SD/-Trp/-Leu/-His master plates. After 3 days, a β -galactosidase filter assay was done.

Antibodies. An anti-hBcl-2 mouse monoclonal antibody (mAb) (clone 100), an anti-hBcl-2 rabbit polyclonal antibody (α -hBcl-2), an anti-cBcl-2 mouse mAb (clone 2A7A3), an anti-mouse Bax rabbit polyclonal antibody (N-20; Santa Cruz), and an anti-HA mouse mAb (clone 12CA5; Boehringer Mannheim) were used. A rabbit polyclonal antibody against SMN (α -SMN) was raised using a peptide corresponding to amino-acid residues 71–84 of SMN. Normal mouse IgG2b (Bethyl), anti-glutathione-S-transferase (GST) rabbit polyclonal IgG (α -GST) (MBL, Japan), and normal rabbit IgG (MBL, Japan) were used as control antibodies for 12CA5, α -hBcl-2 and α -SMN, respectively. An agonistic anti-human Fas mAb CH11 (MBL, Japan) was used to induce apoptosis.

Transfection and immunoprecipitation. pCAGGS-hbcl-2, pCAGGS-HA-SMN, and pUC-CAGGS (vector) in the indicated combinations were transfected into COS-7 cells by electroporation (220 V, 960 μ F). 5 μ g of each plasmid was used to transfect 10⁷ cells. Cells were incubated for 48 h in DMEM supplemented with 10% FBS and collected for immunoprecipitation. After washing twice with PBS, cells were lysed by sonication in lysis buffer (10 mM HEPES, pH 7.5, 142.5 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 0.2% NP-40, 0.1 mM *p*-APMSF, 10 μ g ml⁻¹ aprotinin, 1 μ g ml⁻¹ pepstatin, and 1 μ g ml⁻¹ leupeptin). Cellular debris was removed by centrifugation at 15,000g for 15 min, and lysates were precleared with 5% (v/v) protein G-Sepharose for 60 min and incubated with specific antibodies overnight. Immunoprecipitates were captured with 5% (v/v) protein G-Sepharose for 60 min and washed 5 times in lysis buffer. Immunoprecipitates derived from 10⁶ cells were solubilized with SDS-PAGE sample buffer and subjected to 12.5% SDS-PAGE and western blot analysis.

Microinjection and analysis of nuclear changes. HeLa cells and NT2 cells (Stratagene) were seeded onto glass coverslips with RPMI 1640 medium and OPTI-MEM, respectively, both containing 10% FBS. DNA at the indicated concentrations in PBS was microinjected through a glass capillary into the cell nuclei using a Leica microinjection system. Various amounts of pCAGGS-hbcl-2, pBC140-cbcl-2, pCAGGS-HA-SMN and pCAGGS-BMG plasmids were used for microinjection in various combinations. Cell death of HeLa cells was induced by microinjection of 20 ng μ l⁻¹ of pCAGGS-mbax followed by incubation for 3 h, or by treatment of cells with 1 μ g ml⁻¹ agonistic anti-Fas mAb CH11 for 6 h. Cell death of NT2 cells was induced by co-microinjection of

40 ng μL^{-1} pCAGGS-mbx followed by incubation for 4 h. Total DNA concentrations were adjusted to 200 ng μL^{-1} with DNA of the vector, pUC-CAGGS. BSA (5.8 mg mL^{-1}) labelled with RITC was always co-injected to identify the injected cells. After the indicated periods, cells were fixed with 3.7% formaldehyde for 20 min, stained with 10 μM Hoechst 33342 (Calbiochem) for 5 min, and examined under a fluorescence microscope (Olympus BX-50, Japan) with excitation at 530 nm for RITC and at 360 nm for Hoechst 33342. Apoptotic cells were identified by condensed and fragmented nuclei stained with Hoechst 33342.

Received 17 June; accepted 28 August 1997.

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Acknowledgements. We thank J. Miyazaki, A. Shinohara and D. Y. Mason for their kind gifts of pCAGGS-BMG plasmid, pBTM116-RAD51 and anti-hBcl-2 mAb, respectively; T. Hachiya and M. Ikeda for their help in generating antibodies; and M. Hoffman for editorial assistance. This work was supported in part by grants for Scientific Research on Priority Areas and for COE Research from the Ministry of Education, Science, and Culture of Japan.

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Molecular identification of a volume-regulated chloride channel

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A volume-regulated chloride current ($I_{\text{Cl.vol}}$) is ubiquitously present in mammalian cells, and is required for the regulation of electrical activity, cell volume, intracellular pH, immunological responses, cell proliferation and differentiation. However, the molecule responsible for $I_{\text{Cl.vol}}$ has yet to be determined^{1–3}. Although three putative chloride channel proteins expressed from cloned genes (P-glycoprotein⁴, $p\text{I}_{\text{Cln}}$ (ref. 5) and CIC-2 (ref. 6)) have been proposed to be the molecular equivalent of $I_{\text{Cl.vol}}$, neither P-glycoprotein nor $p\text{I}_{\text{Cln}}$ is thought to be a chloride channel or part thereof^{7,8}, and the properties of expressed CIC-2 channels differ from native $I_{\text{Cl.vol}}$ (refs. 3, 6). Here we report that functional expression in NIH/3T3 cells of a cardiac clone of

another member of the CIC family, CIC-3, results in a large basally active chloride conductance, which is strongly modulated by cell volume and exhibits many properties identical to those of $I_{\text{Cl.vol}}$ in native cells^{1–3,9–13}. A mutation of asparagine to lysine at position 579 at the end of the transmembrane domains of CIC-3 abolishes the outward rectification and changes the anion selectivity from $\text{I}^- > \text{Cl}^-$ to $\text{Cl}^- > \text{I}^-$ but leaves swelling activation intact. Because CIC-3 is a channel protein belonging to a large gene family of chloride channels^{3,14}, these results indicate that CIC-3 encodes $I_{\text{Cl.vol}}$ in many native mammalian cells.

The channel protein encoded by the gene for CIC-3 cloned previously from rat kidney¹⁴, mouse liver and human fetal brain¹⁵, has a similar hydropathy profile (12 putative transmembrane-spanning domains) to other CIC family members³, but uniquely, Cl^- currents expressed from rat CIC-3 in *Xenopus* oocytes are outwardly rectifying, more permeable to I^- than Cl^- , and inhibited by protein kinase C (PKC) and stilbene derivatives such as 4,4'-diisothiocyanostilbene-2,2'-disulphonate (DIDS)¹⁴. These properties are strikingly similar to those of $I_{\text{Cl.vol}}$ in heart^{10–13}, but the possible regulation of CIC-3 by cell volume has not been tested. We cloned the full-length CIC-3 cDNA from guinea-pig heart using a modified reverse transcription–polymerase chain reaction (RT–PCR) technique¹⁶, with the objective of assigning molecular components to cardiac Cl^- channels. Nucleotide sequence homology and amino-acid sequence identity of the resulting guinea-pig cardiac clone (gpCIC-3) were 91.5% and 98.4% with rat kidney CIC-3 (rCIC-3), respectively (GenBank accession no. U83464).

The expression of CIC-3 in guinea-pig heart was confirmed by northern blot analysis. A CIC3-specific probe hybridized to two transcripts (5.1 kb and 3.0 kb) from both atrial and ventricular tissues (Fig. 1A). The RT–PCR reaction of total RNA prepared from the isolated single guinea-pig atrial and ventricular myocytes and specific primers designed to amplify a 712-nucleotide product of gpCIC-3 also confirmed the transcriptional expression of CIC-3 in both atrial and ventricular myocytes (Fig. 1B). PCR performed on cDNA from an RT reaction in which no reverse transcriptase was included did not generate any amplification product. Full-length gpCIC-3 cDNA was then stably transfected into NIH/3T3 cells. Northern hybridization of total RNA isolated from NIH/3T3 cells probed with a gpCIC-3 cDNA specific probe is shown in Fig. 1C; hybridization to a 2.3-kb transcript is apparent in the gpCIC3-transfected cells but not in the untransfected cells.

The tight-seal, whole-cell voltage-clamp and the cell-attached patch-clamp techniques¹⁷ were used to examine the functional expression of CIC-3 channels in these cells. To eliminate possible contamination from endogenous Cl^- currents^{18,19}, parallel control experiments were carefully performed on untransfected NIH/3T3 cells under identical conditions. Under isotonic (300 mOsm per kg H_2O) symmetrical Cl^- (135 mM) conditions, CIC3-transfected cells from four of six independently isolated transfected clones generated large outwardly rectifying whole-cell currents (Fig. 1D, a, d) with current densities of $484 \pm 52 \text{ pA pF}^{-1}$ at +80 mV and $-300 \pm 36 \text{ pA pF}^{-1}$ at –80 mV ($n = 22$). The currents inactivated at positive potentials ($> +80 \text{ mV}$) and reversed ($-1.9 \pm 6 \text{ mV}$, $n = 22$) near the estimated equilibrium potential of Cl^- ($E_{\text{Cl}} = 0 \text{ mV}$ with symmetrical Cl^- , 135 mM). In contrast, only small endogenous whole-cell currents could be detected from untransfected NIH/3T3 cells under the same conditions ($13.4 \pm 4 \text{ pA pF}^{-1}$ at +80 mV and $-24 \pm 6 \text{ pA pF}^{-1}$ at –80 mV, $n = 35$, $P < 0.001$ versus CIC3-transfected cells) (Fig. 1E, a). These results indicate that there is stable and functional expression of the gpCIC3 cDNA in the transfected cells.

Because the basally active Cl^- current ($I_{\text{Cl.b}}$) in heart is regulated by cell volume^{10,11}, the possible regulation by volume of basally active $I_{\text{CIC-3}}$ in transfected NIH/3T3 cells was examined by changing the extracellular osmolarity. When the same CIC-3-transfected cell was further superfused with hypotonic solution (250 mOsm per kg

H₂O, 17% hypotonic) for >2 min, significant cell swelling was observed and the membrane conductance dramatically increased (Fig. 1D, b). Similar results were obtained in 21 other cells ($980 \pm 123 \text{ pA pF}^{-1}$ at +80 mV and $-651 \pm 109 \text{ pA pF}^{-1}$ at -80 mV, $n = 22$). Currents activated by cell swelling also exhibited strong outward rectification and time- and voltage-dependent inactivation at positive potentials (>+80 mV). The total cell-swelling-activated current density in ClC-3-transfected cells was about twofold greater than the basally active current recorded in these cells under isotonic conditions ($P < 0.001$).

Endogenous $I_{\text{Cl,swell}}$ has been reported in NIH/3T3 cells^{18,19}, so we examined the effects of a similar 17% hypotonic solution on current amplitudes of parallel-controlled untransfected cells. Cell swelling caused a small increase in current amplitudes in these cells with current densities ($63 \pm 17 \text{ pA pF}^{-1}$ at +80 mV and $-44 \pm 10 \text{ pA pF}^{-1}$ at -80 mV, $n = 35$) in the same range as those of endogenous $I_{\text{Cl,swell}}$ reported previously^{4,18,19} (Fig. 1E, b), but about 16-fold smaller ($P < 0.001$) than those observed in 17% hypotonic solutions in ClC-3-transfected cells. As a further control, NIH/3T3 cells transfected with pZeoSV-CFTR were examined for the presence of ClC-3-like currents. Although cAMP-dependent Cl⁻ currents could be recorded, consistent with CFTR expression, currents similar to those recorded from ClC-3-transfected cells could not be detected (data not shown). This control provides evidence that stably transfecting NIH/3T3 cells to Zeocin resistance does not result in the induction of endogenous volume-regulated Cl⁻ currents. These results strongly indicate that the increase in the current density induced by hypotonicity in ClC-3-transfected cells is attributable to expression of gpClC-3 protein. Furthermore, under hypertonic conditions (350 mOsm per kg H₂O, 117% hypertonic), the current remaining in ClC-3-transfected cells, although significantly reduced (Fig. 1D, c, d), was still about threefold larger ($202 \pm 24 \text{ pA pF}^{-1}$ at +80 mV and $-138 \pm 10 \text{ pA pF}^{-1}$ at -80 mV, $n = 5$) than the total endogenous $I_{\text{Cl,swell}}$ recorded in untransfected cells under hypotonic conditions ($P < 0.001$).

Further evidence that gpClC-3 may be responsible for $I_{\text{Cl,vol}}$ was provided by examining the pharmacological properties of whole-cell $I_{\text{ClC-3}}$ in ClC-3-transfected NIH/3T3 cells. Consistent with the characteristic inhibition by PKC of both native cardiac $I_{\text{Cl,vol}}$ (ref. 10) and cloned rClC-3 (refs 14,20), activation of PKC by phorbol 12,13-dibutyrate (PDBu, 100 nM) inhibits both basally active (Fig. 2A) and swelling-activated (Fig. 2B) $I_{\text{ClC-3}}$ in a voltage-independent manner ($74 \pm 8\%$ block at -80 mV and $76 \pm 7\%$ block at +80 mV for basally active current, n.s., $n = 3$; $79 \pm 5\%$ block at -80 mV and $78 \pm 5\%$ block at +80 mV for swelling-activated current, n.s., $n = 5$). Moreover, DIDS (100 μM ; Fig. 2C), the anti-oestrogen compound tamoxifen (10 μM ; Fig. 2D), and extracellular nucleotides (ATP_o, 10 mM; Fig. 2E) all blocked $I_{\text{ClC-3}}$ with a characteristic voltage dependence that closely resembles their effects on $I_{\text{Cl,vol}}$ in cardiac¹⁰⁻¹³ and other mammalian cells^{1-3,9-19}. The notion that the cell-swelling-activated current in ClC-3-transfected cells was due to activation of the same type of channel as seen under the basal condition was further supported by their identical anion selectivity of $\text{I}^- > \text{Cl}^- > \text{Asp}^-$ (Fig. 2F).

Representative single-channel properties of $I_{\text{ClC-3}}$ recorded in cell-attached patches of gpClC-3-transfected cells are shown in Fig. 3. Consistent with the low endogenous current density observed in whole-cell experiments, single-channel currents could not be detected in cell-attached patches from untransfected cells under isotonic (23 patches) or hypotonic conditions (18 patches). In ClC-3-transfected cells, intermediate-conductance ($\sim 40 \text{ pS}$) outwardly rectifying Cl⁻ channels were observed in 9 of 28 (32%) patches under isotonic conditions and in 10 of 27 (37%) patches under hypotonic conditions. Although the same percentage of patches containing active channels was observed under the two conditions, the prevalence of active channels in hypotonic solutions was greater (63%) than in isotonic solutions (32%), as there was a larger

($P < 0.05$) total number of active channels in the patches under hypotonic conditions (Fig. 3C). Channels recorded under the two conditions exhibited identical outward rectification, chord conductances and Cl⁻ sensitivity (Fig. 3A, B). The open-channel probability (P_o) of the channel did not exhibit any obvious voltage dependence (0.598, 0.716, 0.616, 0.713, 0.680 and 0.691 at 20 mV below the resting potential (RP-20), RP, RP + 20, RP + 40, RP + 60 and RP + 80 mV, respectively; not significant for voltage dependence, ANOVA). Unconditional kinetic analysis^{11,20,21} of open and closed times of single channels indicated a minimum of three open kinetic states ($\tau_1 = 1.1 \text{ ms}$, $\tau_2 = 8.3 \text{ ms}$, $\tau_3 = 83.0 \text{ ms}$) and four closed kinetic states ($\tau_1 = 0.3 \text{ ms}$, $\tau_2 = 2.6 \text{ ms}$, $\tau_3 = 19.8 \text{ ms}$, $\tau_4 = 152.9 \text{ ms}$) (Fig. 3D, c, d). These single-channel properties, including the outward rectification, slope conductance, voltage independence of P_o , kinetics, and the regulation by cell volume are nearly identical to those of $I_{\text{Cl,vol}}$ in native cardiac myocytes¹¹. However, these properties differ significantly from those obtained for rClC-3 channels in cell-free membrane patches of rClC-3-transfected Chinese hamster ovary cells²⁰. The reason for this discrepancy is unclear.

To ensure that gpClC-3 was responsible for the expressed channel and currents recorded in transfected NIH/3T3 cells, we introduced a mutation in the gpClC-3 cDNA that encodes an asparagine at the end of the transmembrane spanning domains (Fig. 4A), as the corresponding amino acid has been shown to be important for both pore properties and gating in ClC-0 (K519)²²⁻²⁴ and ClC-2 (K566)²⁵. The neutral asparagine 579 was changed to positively charged lysine,

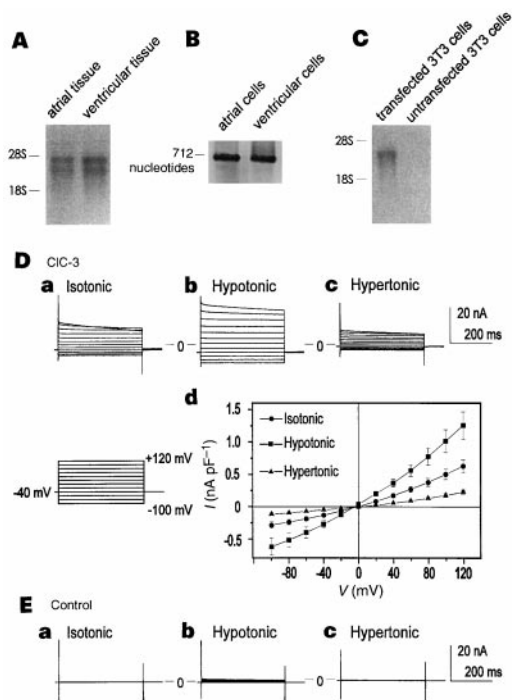


Figure 1 Expression of ClC-3 in guinea-pig heart. **A**, Northern hybridization of RNA derived from guinea-pig atrial and ventricular tissues. **B**, Agarose gel depicting ClC-3 specific RT-PCR products generated from RNA derived from enzymatically dispersed atrial and ventricular myocytes. **C**, Northern analysis of total RNA derived from gpClC-3-transfected and untransfected NIH/3T3 cells. **D**, **E**, Current-voltage (I - V) relation and volume regulation of whole-cell currents recorded from gpClC-3-transfected (**D**) and untransfected (**E**) NIH/3T3 cells under isotonic (**a**), hypotonic (**b**) and hypertonic (**c**) conditions. Cells were clamped from a holding potential of -40 mV in +20 mV steps from -100 to +120 mV for 400 ms at an interval of 5 s (inset in **D**). **D**, **d**, Mean I - V curves of $I_{\text{ClC-3}}$ under isotonic (circles, $n = 22$), hypotonic (squares, $n = 22$), and hypertonic (triangles, $n = 5$) conditions. The current amplitudes (nA) were measured at 8 ms after the corresponding voltage step relative to the 0 current level and then normalized to the capacitance (pF) of the cell.

and the N579K gpCIC-3 cDNA was transfected into NIH/3T3 cells. To eliminate possible endogenous $I_{\text{CL,swell}}$ contamination caused by Zeocin or clone selection, we altered the method of obtaining transfected cells expressing the CIC-3 channel from stable transfection to transient transfection, and compared the properties of wild-type and mutant N579K CIC-3 channels under identical conditions. Only small $I_{\text{CL,swell}}$ was detected in CD8 plasmid-transfected control cells (Fig. 4B), but large outwardly rectifying volume-sensitive currents with properties identical to those observed in CIC-3 stably transfected cells were observed in wild-type CIC-3 transiently transfected cells (Fig. 4C). Compared with wild-type CIC-3, mutated channels were no longer outwardly rectifying, rather they showed a slight inward rectification (Fig. 4D). Although N579K channels were still basally active and potentiated by cell swelling,

anion selectivity of the channel changed from $\text{I}^- > \text{Cl}^-$ (Fig. 4E) to $\text{Cl}^- > \text{I}^-$ (Fig. 4F).

Taken together, these data demonstrate that the CIC-3 gene encodes a volume-regulated Cl^- channel and its macroscopic equivalents, $I_{\text{CL,b}}$ and $I_{\text{CL,swell,b}}$ in heart. It is probably also responsible

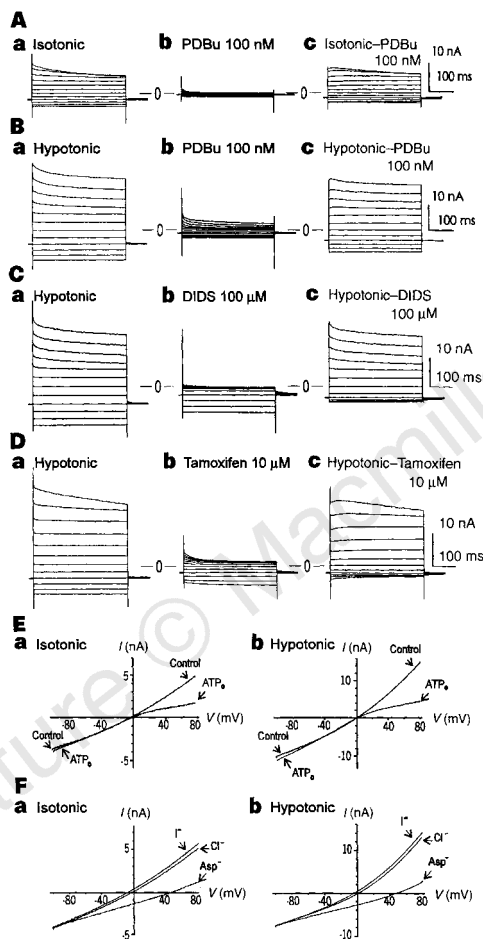


Figure 2 Pharmacology and ion selectivity of $I_{\text{CIC-3}}$ in NIH/3T3 cells. **A, B**, PKC under isotonic (**A**) and hypotonic (**B**) conditions. **C**, Extracellular DIDS (100 μM) inhibited the swelling-activated $I_{\text{CIC-3}}$ in a voltage-dependent manner (95 $\pm$ 2% block at +80 mV and 37 $\pm$ 3% block at -80 mV, $n = 3$, $P < 0.001$). **D**, Tamoxifen (10 μM) also inhibited swelling-activated $I_{\text{CIC-3}}$ in a voltage-dependent manner (82.1 $\pm$ 1% block at +80 mV and 45 $\pm$ 9% block at -80 mV, $n = 3$, $P < 0.05$). In **A-D**, **c**, represents the difference currents before and after each treatment. The voltage-clamp protocol is the same as shown in Fig. 1D. **E**, A ramp voltage-clamp protocol (depolarization rate = 0.2 V s^{-1} , from -100 to +80 mV at a holding potential of -40 mV) was used to study extracellular nucleotide (ATP_o) regulation of $I_{\text{CIC-3}}$. ATP_o (10 mM) inhibited only outward current of the basally active (**a**) and swelling-activated (**b**) $I_{\text{CIC-3}}$. **F**, I - V relations of whole-cell currents (ramp protocol was the same as in **E**) in CIC3-transfected cells before and after $[\text{Cl}^-]_o$ was replaced by equimolar (110 mM) I^- or Asp^- ($E_{\text{Cl}} = +42.8 \text{ mV}$). I^- and Asp^- substitution of $[\text{Cl}^-]_o$ shifted the reversal potential (E_{rev}) of basal $I_{\text{CIC-3}}$ (**a**) from $-1.3 \pm 2.1 \text{ mV}$ to $-7.8 \pm 2.7 \text{ mV}$ and $+42.2 \pm 3.0 \text{ mV}$ ($n = 4$), respectively, and of swelling-activated $I_{\text{CIC-3}}$ (**b**) from $-2.1 \pm 0.9 \text{ mV}$ to $-10.8 \pm 0.6 \text{ mV}$ and $+46.7 \pm 1.9 \text{ mV}$ ($n = 4$), respectively.

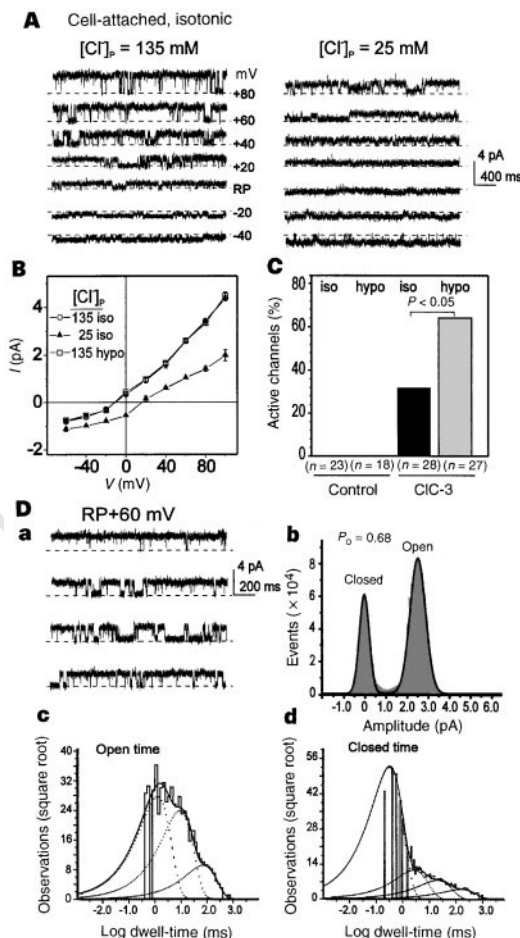


Figure 3 Single-channel properties of $I_{\text{CIC-3}}$ in cell-attached patches from gpCIC-3-transfected NIH/3T3 cells. **A**, Representative current traces at various potentials with different pipette Cl^- concentration $[\text{Cl}^-]_p$ under isotonic conditions. The membrane was clamped from a holding potential of 0 mV relative to the resting potential (RP) to a series of test potentials imposed at 0.1 Hz in +20 mV increments from RP - 60 to RP + 100 mV for 2 s. **B**, Mean I - V curves of basally active and swelling-activated unitary $I_{\text{CIC-3}}$. With $[\text{Cl}^-]_p = 135 \text{ mM}$ (open circles, $n = 5$), single-channel currents recorded under isotonic conditions showed strong outward rectification with chord conductance of $43.1 \pm 0.7 \text{ pS}$ at RP + 60 mV and $12.9 \pm 0.6 \text{ pS}$ at RP - 60 mV and reversed at RP - 16.3 $\pm$ 1.6 mV. The average slope conductance was $39.7 \pm 0.6 \text{ pS}$ (outward current from RP to RP + 100 mV) and $12.8 \pm 1.8 \text{ pS}$ (inward current from RP - 20 to RP - 60 mV). With $[\text{Cl}^-]_p = 25 \text{ mM}$ (filled triangles, $n = 4$), slope conductance of outward current (RP + 20 to RP + 100 mV) was $22.1 \pm 3.8 \text{ pS}$ and E_{rev} was RP + 14.4 $\pm$ 1.8 mV. The average slope conductance of swelling-activate single-channels at $[\text{Cl}^-]_p$ of 135 mM (open squares, $n = 3$) was $40.4 \pm 1.5 \text{ pS}$ (outward current from RP to RP + 100 mV) and $10.8 \pm 1.6 \text{ pS}$ (inward current from RP - 20 to RP - 60 mV) and E_{rev} was RP - 15.8 $\pm$ 1.9 mV (not significant compared with isotonic condition). **C**, Prevalence of active channels (total active channel/total tested patches $\times$ 100%) in CIC-3-transfected and untransfected NIH/3T3 cells under isotonic and hypotonic conditions. **D**, Open probability and kinetic analysis of unitary $I_{\text{CIC-3}}$ at RP + 60 mV. **a**, Representative current traces. **b**, All-point amplitude histogram. Solid curve shows the gaussian fits, indicating only one active channel in this patch. **c, d**, Open (**c**) and closed (**d**) dwell-time histograms. The interval durations were log-binned and the number of events in each interval was transformed to square-root. Broken lines indicate the fitting components, and the solid line indicates the final fitting curve.

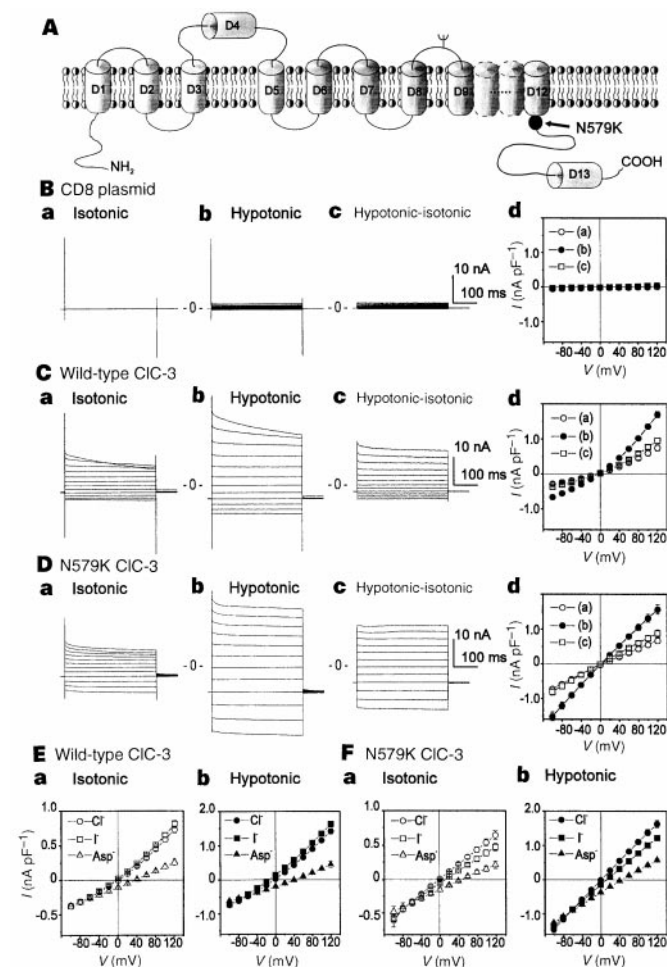


Figure 4 Properties of wild-type and mutant N579K gpCIC-3 transiently expressed in NIH/3T3 cells. **A**, Transmembrane topology model for CIC-3 channels³ and the location of mutated residue in gpCIC-3. **B–D**, Volume regulation of whole-cell currents and mean I - V relations recorded in NIH/3T3 cells transiently transfected with CD8 plasmid alone (**B**), wild-type (**C**), and mutant N579K gpCIC-3 cDNA (**D**). Cl^- concentrations and voltage-clamp protocols are the same as in Fig. 1. Traces shown are current traces recorded under isotonic (**a**) and hypotonic (**b**) conditions, the difference currents before and after cell swelling (**c**), and the mean I - V curves of each (**d**). Under isotonic conditions, only small currents were detected in CD8 plasmid-transfected control cells ($5.4 \pm 4.2 \text{ pA pF}^{-1}$ at $+80 \text{ mV}$ and $-28.4 \pm 4.5 \text{ pA pF}^{-1}$ at -80 mV , $E_{\text{rev}} = 22.0 \pm 3.4 \text{ mV}$, $n = 8$), whereas large outwardly rectifying currents were observed in wild-type CIC-3 transiently transfected cells ($439 \pm 48 \text{ pA pF}^{-1}$ at $+80 \text{ mV}$ and $-247 \pm 29 \text{ pA pF}^{-1}$ at -80 mV , $E_{\text{rev}} = -2.8 \pm 0.5 \text{ mV}$, $n = 7$). Large currents were also observed in N579K CIC-3-transfected cells but the I - V relations were inwardly rectifying ($450 \pm 61 \text{ pA pF}^{-1}$ at $+80 \text{ mV}$ and $-567 \pm 53 \text{ pA pF}^{-1}$ at -80 mV , $E_{\text{rev}} = 3.3 \pm 0.8 \text{ mV}$, $n = 6$). Hypotonic cell swelling significantly increased current densities in both wild-type ($1,011 \pm 46 \text{ pA pF}^{-1}$ at $+80 \text{ mV}$ and $-569 \pm 40 \text{ pA pF}^{-1}$ at -80 mV , $E_{\text{rev}} = -3.9 \pm 0.7 \text{ mV}$, $n = 7$) and N579K ($1,065 \pm 89 \text{ pA pF}^{-1}$ at $+80 \text{ mV}$ and $-1,194 \pm 98 \text{ pA pF}^{-1}$ at -80 mV , $E_{\text{rev}} = 1.4 \pm 7 \text{ mV}$, $n = 6$) gpCIC-3-transfected cells. Only a small increase in membrane currents could be detected in CD8 plasmid-transfected control cells ($40 \pm 6 \text{ pA pF}^{-1}$ at $+80 \text{ mV}$ and $-13 \pm 4 \text{ pA pF}^{-1}$ at -80 mV , $E_{\text{rev}} = -6.0 \pm 4.7 \text{ mV}$, $n = 8$). **E, F**, Mean I - V curves of wild-type (**E**) and N579K (**F**) $I_{\text{CIC-3}}$ under isotonic (**a**) and hypotonic (**b**) conditions before and after $[\text{Cl}^-]_0$ was replaced (see Fig. 2F). **E**, I^- and Asp^- shifted E_{rev} of basal wild-type $I_{\text{CIC-3}}$ (**a**) from $-1.4 \pm 0.5 \text{ mV}$ to $-8.6 \pm 1.4 \text{ mV}$ and $+38.2 \pm 2.5 \text{ mV}$ ($n = 5$), respectively, and of swelling-activated wild-type $I_{\text{CIC-3}}$ (**b**) from $-1.9 \pm 0.4 \text{ mV}$ to $-13.9 \pm 0.5 \text{ mV}$ and to $+44.7 \pm 3.6 \text{ mV}$ ($n = 7$), respectively. **F**, replacement of $[\text{Cl}^-]_0$ shifted E_{rev} of basal N579K $I_{\text{CIC-3}}$ (**a**) from $1.6 \pm 0.7 \text{ mV}$ to $+17.3 \pm 2.3 \text{ mV}$ ($n = 5$) and of swelling-activated N579K $I_{\text{CIC-3}}$ (**b**) from $1.9 \pm 0.5 \text{ mV}$ to $+15.3 \pm 0.9 \text{ mV}$ ($n = 5$), indicating that the anion selectivity of both the basally active and swelling-activated N579K $I_{\text{CIC-3}}$ was changed to $\text{Cl}^- > \text{I}^- > \text{Asp}^-$, compared with $\text{I}^- > \text{Cl}^- > \text{Asp}^-$ for the wild type.

for a similar channel in other types of cells, because CIC-3 is widely expressed in a variety of tissues of different species including human^{14,15}. The biophysical and pharmacological properties of the expressed $I_{\text{CIC-3}}$ were nearly identical to those reported for native $I_{\text{Cl.vol}}$ in a variety of tissues, and included a unitary slope conductance of $\sim 40 \text{ pS}$, an anion selectivity of $\text{I}^- > \text{Cl}^- > \text{Asp}^-$, inactivation at positive potentials, an increase in extracellular hypotonicity, and inhibition by hypertonicity, extracellular nucleotides, phorbol esters, stilbene derivatives and tamoxifen. Moreover, a specific mutation in CIC-3 dramatically altered the anion-selectivity and rectification properties of the expressed channels. These data effectively exclude the possibility that CIC-3 expression is merely upregulating the activity of endogenous swelling-activated Cl^- channels in the NIH/3T3 expression system, and provide direct evidence that the CIC-3 gene encodes a volume-regulated Cl^- channel. However, our data do not exclude the possibility that the volume-regulated Cl^- channel in native cells may actually be a heterodimer composed of CIC-3 along with another CIC subunit²⁶. Future functional and mutational studies of the CIC-3 gene product should provide important new clues to the molecular link between changes in cell volume and channel activation. □

Methods

Cloning and stable functional expression of guinea-pig cardiac CIC-3. The guinea-pig cardiac CIC-3 cDNA was isolated using a modified RT-PCR method¹⁶. Several overlapping primer oligonucleotides were used. Oligo dT and specific primers were designed to hybridize to sequence in the 3' region. Oligonucleotides corresponding to nucleotide positions 1190–1212 and 2275–2297 in the rat sequence were used as 3' anchor-reverse primers in conjunction with primers (in individual reactions) encoding regions of the rCIC-3 sequence¹⁴ for the 5' forward primers corresponding to nucleotide positions

–6–17 and 500–522 in the rat sequence. Poly(A)⁺ RNA prepared from ventricular and atrial muscles (0.3 g) of several animals by using the Fast Track kit (Invitrogen, San Diego, CA) was applied to a denaturing agarose gel and separated on the basis of molecular weight by electrophoresis. The RNA was transferred to hydrophobic filters and qualitative northern blot hybridizations were carried out using a radiolabelled cDNA probe for gpCIC-3. A 550-nucleotide *EcoRI* was prepared from the clone and labelled with ³²P using a random-primer technique²⁷. After hybridization, filters were exposed to screens and autoradiographed using a BioRad (Hercules, CA) phosphorimager. The RT-PCR of total RNA prepared from isolated atrial and ventricular myocytes²⁸ was performed using interior primers as described for cDNA cloning. The region is specific for CIC-3 and corresponds to nucleotides 500–1212 generating a 712-nucleotide amplification product. Automatic nucleotide sequencing was performed on both strands by the dideoxy nucleotide chain termination method (Applied Biosystems, model 310). Full-length gpCIC-3 was ligated to the mammalian expression vector pZeoSV (Invitrogen) and transfected into NIH/3T3 cells by electroporation. The asparagine at position 579 was altered to lysine by a site-specific mutation in gpCIC-3 cDNA²⁹. The mutation was confirmed by nucleotide sequencing of both strands of the mutated cDNA. NIH/3T3 cells were transiently transfected by electroporation. Each dish was transfected with appropriate combinations of the following: CD8 (a lymphocyte cell-surface antigen) in the $\pi\text{H3-CD8}$ plasmid construct as a marker for transfection ($4 \mu\text{g}$)³⁰; gpCIC-3 or N579K gpCIC-3 in the pZeoSV vector ($10 \mu\text{g}$). Transfected cells were identified by their binding to CD8-coated beads (Dyna-beads M-450 CD8; Great Neck, NY). Cells were subcultured on glass coverslips for electrophysiological recording.

Whole-cell and single-channel recordings. Currents were measured from isolated NIH/3T3 cells at room temperature (22 – 24°C) by the tight-seal, whole-cell voltage-clamp or cell-attached patch-clamp techniques^{10,11,17}. Bath and pipette solutions were chosen to facilitate Cl^- current recording. The hypotonic ($250 \text{ mOsm per kg H}_2\text{O}$, measured by freezing-point depression)

bath (external) solutions contained (in mM): NaCl 125, MgCl₂ 2.5, CaCl₂ 2.5, HEPES 10, pH 7.2; [Cl⁻]_o = 135 mM. The isotonic and hypertonic bath solutions were the same as the hypotonic solution except that the osmolarity was adjusted to 300 and 350 mOsm per kg H₂O by adding mannitol. The pipette (internal) solution for whole-cell recordings contained (in mM): N'-methyl-D-glucamine chloride (NMDG-Cl) 135, EGTA 2, Mg-ATP 5, HEPES 10, pH 7.2; [Cl⁻]_i = 135 mM, 300 mOsm per kg H₂O by adding mannitol. The pipette (external) solution for cell-attached single-channel recordings contained (in mM): NMDGCl 135 (or NMDG-Cl 25, NMDG-Aspartate 110), HEPES 5, glucose 5.5, pH 7.2. The cell-attached patch configuration was checked at the end of each experiment by rupturing the patch to confirm a passage from the cell-attached to the whole-cell configuration. Intracellular potentials of cells were immediately measured right after this step. The average intracellular potential measured from 51 cells was -19 ± 2 mV. For the analysis of P_o and the open and closed kinetics, patches were held at the desired potential for at least 5 min. Kinetics of open and closed events were analysed for patches containing only one active channel (determined by all-points amplitude histogram) using the methods described previously^{11,20,21}.

Received 28 July; accepted 1 September 1997.

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Acknowledgements. We thank N. Horowitz, F. Schmalz, L. Ye and T. Truong for technical support, and J. Yamazaki for assistance in experiments with pZeoSV-CFTR. This study was supported by the Medical Research Council of Canada (D.D.), the American Heart Association, Nevada Affiliate (C.W.), and the NIH (J.R.H. and B.H.).

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Mobility of photosynthetic complexes in thylakoid membranes

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The structures of many photosynthetic pigment–protein complexes have now been determined^{1–6}, but a real understanding of the photosynthetic membrane at the molecular level will also require knowledge of the organization and dynamics of these complexes in the intact membrane. Using fluorescence recovery after photobleaching (FRAP)⁷ and a scanning confocal microscope, we have made direct measurements *in vivo* of the lateral diffusion of light-harvesting complexes and reaction centres in the thylakoid membranes of the cyanobacterium *Dactylococcopsis salina*⁸. We find that the phycobilisomes (the accessory light-harvesting complexes of cyanobacteria) diffuse quite rapidly, but that photosystem II is immobile on the timescale of the measurement, indicating that the linkage between phycobilisomes and photosystem II is unstable. We propose that the lateral diffusion of phycobilisomes is involved in regulation of photosynthetic light-harvesting (state 1–state 2 transitions). The mobility of the phycobilisomes may also be essential to allow the synthesis and repair of thylakoid membrane components.

FRAP has been widely used for measuring the lateral diffusion of components of eukaryotic cell membranes⁹. Typically, mammalian cells with large, flat surfaces are used. The components to be studied are labelled with a fluorescent tag, and a focused laser beam is used to bleach an area of the labelled membrane. The recovery of fluorescence in the bleached area is monitored with the same laser. This indicates the rate at which unbleached chromophores diffuse into the bleached area. A refinement of the technique uses a scanning confocal microscope: the beam is scanned over a small area of the cell surface to bleach the chromophore and then over a larger area to record a sequence of images¹⁰.

The long-range diffusion coefficient for LHCII, the integral membrane light-harvesting complex of green plants, has been estimated from rapid membrane-fractionation studies¹¹. However, there has been no direct measurement of the mobility of photosynthetic complexes in a native membrane. Photosynthetic pigment–protein complexes contain fluorescent chromophores, so there is no need for an added fluorescent label in a FRAP measurement. We do not know of any photosynthetic membrane with the extended flat surface required for conventional FRAP measurements. However, the cyanobacterium *Dactylococcopsis salina* has unusually large cells (typically about 4–8 μ m across and 35–80 μ m long)⁸. As in many cyanobacteria, the thylakoid membranes are concentric cylinders aligned along the long axis of the cell (A. E. Walsby, personal communication). We have exploited the rotational symmetry of the cells by bleaching a plane across the short axis of the cell. This generates a one-dimensional bleaching profile along the long axis of the cell (Fig. 1). The evolution of the bleaching profile can be interpreted in terms of the one-dimensional diffusion equation (Box 1).

Cyanobacteria are prokaryotes that perform oxygenic photosynthesis. Like green plants, cyanobacteria possess the chlorophyll-containing photosystem I and photosystem II reaction centres¹². The accessory light-harvesting complexes of cyanobacteria are the phycobilisomes, which are aggregates of phycobiliproteins anchored to the cytoplasmic surface of the thylakoid

membrane². Phycobilisomes are typically hemidiscoidal and ~70 nm in diameter, with a relative molecular mass of 4,500K–15,000K (ref. 2). Phycobilisomes are not present in green plants, which use integral membrane chlorophyll-binding proteins instead⁴.

FRAP was measured using a scanning confocal microscope with two lasers as alternative light sources (see Methods). The 442 nm laser excites chlorophyll *a* of photosystem I and photosystem II. Only photosystem II fluoresces significantly at room temperature¹³, so essentially all the fluorescence detected is from photosystem II. The 633 nm laser excites the phycocyanin and allophycocyanin chromophores in phycobilisomes². Fluorescence excitation and emission spectra for *Dactylococcopsis* cells (not shown) are typical for cyanobacteria. In the excitation spectrum, there is a small peak at 440 nm (chlorophyll *a*) and larger peaks at 580–625 nm (phycocyanin²). There is no indication of any unusual, highly fluorescent chromophores which might be detected in our measurements in addition to the phycobilisome components and the chlorophylls of photosystem II.

Figure 2 shows typical sequences of fluorescence images. The derived bleaching profiles are shown in Fig. 3. With phycobilisome excitation (Figs 2a and 3a), the initial bleaching profile is gaussian, with a half-width ($1/e^2$) of 2.5 μm . The bleaching is much broader than the confocal spot (about 0.3 μm): this must be due to scattering of the bleaching light by the multiple layers of membrane. The initial width and depth of the bleach varied in different measurements (Table 1). At the centre of the bleach, 70–80% of fluorescence was normally lost (Table 1). We are therefore measuring the behaviour of the bulk population of phycobilisomes, not a minor subpopulation or a chromophore not associated with the phycobilisomes. Within a few minutes, the bleaching profile spreads, becoming broader and shallower. This shows that phycobilisomes are diffusing, with unbleached phycobilisomes diffusing into the bleached area (Figs 2a and 3a).

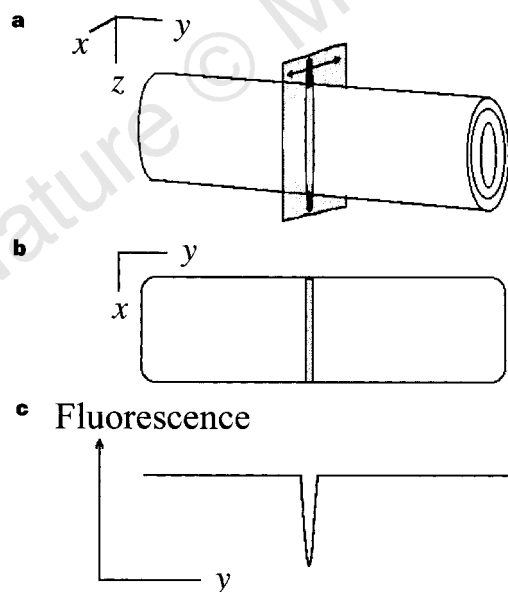


Figure 1 The geometry of our one-dimensional FRAP measurements. **a**, The objective of the confocal microscope produces an illumination spot elongated in the *z* direction. Scanning the spot in the *x* direction along the short axis of the cell causes photobleaching in the *x*-*z* plane. **b**, The fluorescence from the cell is imaged by scanning the confocal spot in the *x*-*y* plane. The reduced optical resolution in the *z* direction means that the measured signal is the total fluorescence from the full depth of the cell in the *z* direction. The image shows a photobleached line across the cell in the *x* direction. **c**, The image is integrated across the full width of the cell in the *x* direction to give a one-dimensional bleaching profile (fluorescence versus position on the long axis of the cell).

Box 1. The one-dimensional diffusion equation

The one-dimensional diffusion equation is

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial y^2} \quad (1)$$

where *t* is time, *y* is distance along the long axis of the cell, $C_{(y,t)}$ is the concentration of the bleached chromophore and *D* is the diffusion coefficient. If the initial bleaching profile is gaussian, so that: $C_{(y,t=0)} = C_{(y=0,t=0)} \exp[-2y^2/R_0^2]$, where R_0 is the half-width ($1/e^2$) of the bleach, which is centred at *y* = 0, then the solution of equation (1) (assuming that the cell is very long in comparison to the width of the bleach) is: $C_{(y,t)} = C_{(y=0,t=0)} R_0 (R_0^2 + 8Dt)^{-1/2} \exp[-2y^2/(R_0^2 + 8Dt)]$. Note that the bleaching profile remains gaussian, but becomes broader and shallower with time.

At time *t*, the half-width ($1/e^2$) of the bleach is given by: $R_{(t)} = [R_0^2 + 8Dt]^{1/2}$, and the peak concentration of bleached chromophore is given by: $C_{(y=0,t)} = C_{(y=0,t=0)} R_0 [R_0^2 + 8Dt]^{-1/2}$.

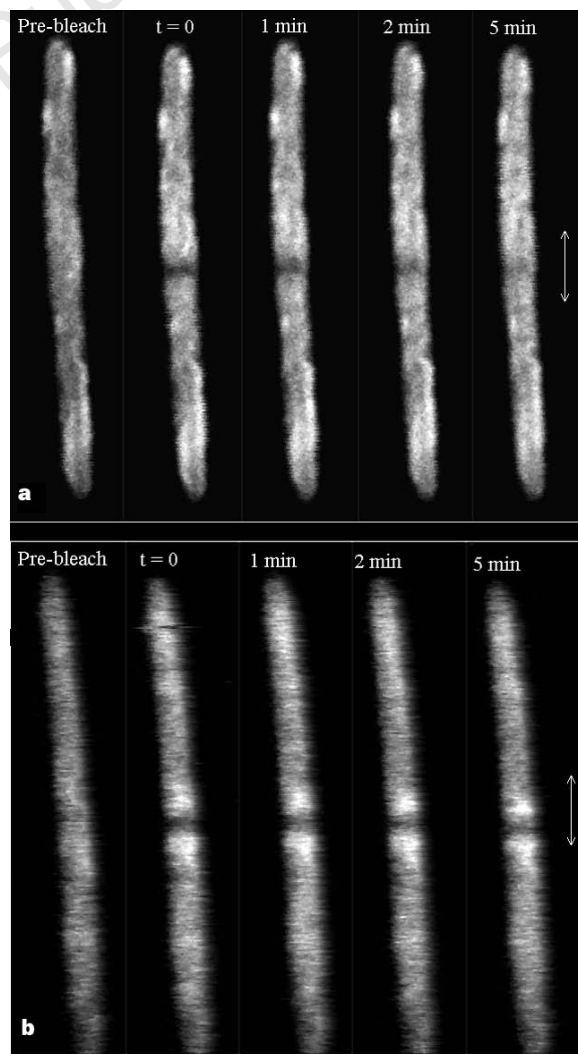


Figure 2 Selected fluorescence images from typical sequences recorded before bleaching, immediately after bleaching, and at various times thereafter. **a**, Excitation at 633 nm, showing fluorescence from the phycobilisomes. **b**, Excitation at 442 nm, showing fluorescence from photosystem II. Scale bars, 10 μm .

Table 1 Diffusion coefficients for phycobilisomes in different cells

Experiment	Maximum initial bleaching*	R_0 (μm)	D ($\text{cm}^2 \text{s}^{-1}$)
1	0.78	2.51 ± 0.06	$(6.3 \pm 0.5) \times 10^{-11}$
2	0.80	3.94 ± 0.10	$(17.3 \pm 0.6) \times 10^{-11}$
3	0.63	3.02 ± 0.17	$(22.1 \pm 3.0) \times 10^{-11}$
4	0.75	3.49 ± 0.09	$(18.8 \pm 1.0) \times 10^{-11}$
5	0.77	3.48 ± 0.13	$(5.6 \pm 0.9) \times 10^{-11}$

In each case, the diffusion coefficient was calculated as for Fig. 4.

*Fraction of fluorescence bleached.

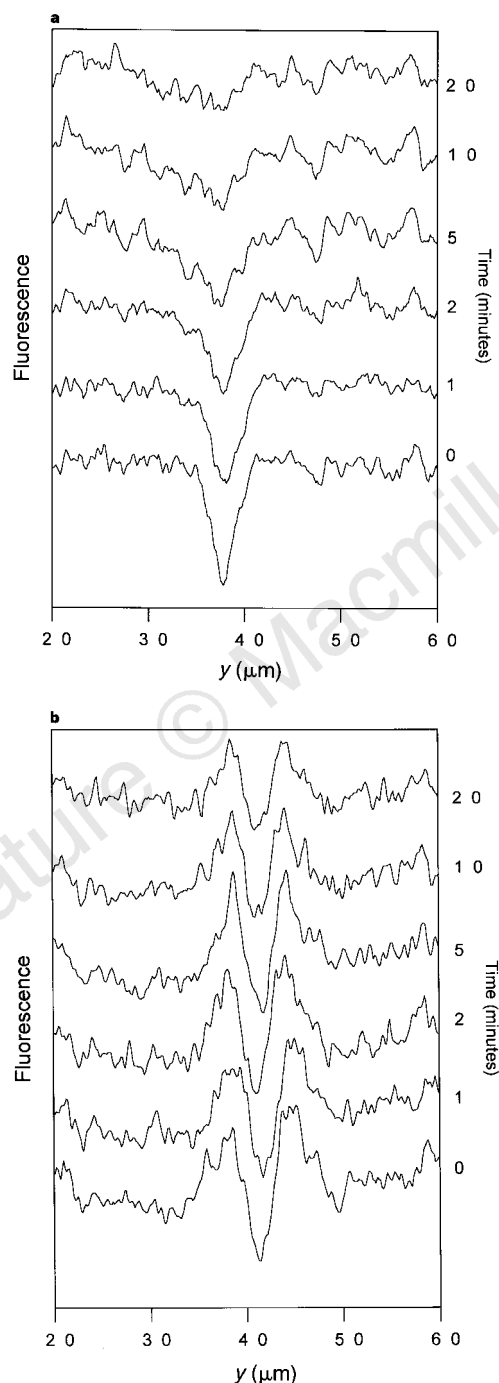


Figure 3 Selected one-dimensional bleaching profiles derived from the sequences of fluorescence images shown in Fig. 2. **a**, Excitation at 633 nm (phycobilisome fluorescence). **b**, Excitation at 442 nm (photosystem II fluorescence).

With photosystem II excitation, the initial bleaching profile is complex: a central bleached zone is flanked by areas of increased fluorescence (Figs 2b and 3b). The fluorescence increase must be the result of mild exposure to the scattered bleaching laser. Light exposure sufficient to disrupt photochemistry without bleaching a significant proportion of the chlorophyll would cause a fluorescence increase. The bleaching profile evolves very little on the timescale of the measurement. At longer times (10–20 min), the bleach becomes shallower. There is no corresponding spread of the bleach, suggesting that *in situ* processes are occurring rather than diffusion. In eukaryotic cells, immobility of membrane proteins is generally ascribed to attachment to the cytoskeleton⁹. There is no evidence for a cytoskeleton capable of performing a similar role in cyanobacteria. However, photosystem II complexes in cyanobacteria are frequently arranged in long parallel rows^{6,14,15}. Tight coupling to such structures may prevent diffusion.

The diffusion coefficient can be obtained from the evolution of the depth of the bleach (Box 1). This calculation for phycobilisomes is shown in Fig. 4. The independent calculation from the spread of the bleach gives a similar result of $(5.4 \pm 0.7) \times 10^{-11} \text{ cm}^2 \text{s}^{-1}$ (not shown). Together with the linearity of the plot of Fig. 4, this indicates that our measurement does not greatly perturb the system. Phycobilisomes diffuse at similar rates in the originally bleached area and in the adjacent unbleached areas of the membrane. Experiments on different cells gave a range of values for D (Table 1). The diffusion coefficients are within the range typical for 'slow' membrane proteins, with diffusion slowed by transient binding to immobile components⁹. The rate of diffusion of phycobilisomes could be limited either by transient binding to immobile reaction centres in the membrane, or by steric hindrance in the cytoplasm. The diffusion coefficient for phycobilisomes is considerably greater than that estimated for the integral membrane light-harvesting complex of green plants¹¹.

The association of phycobilisomes with photosystem II must be transient and unstable, because phycobilisomes diffuse whereas photosystem II is immobile. Efficient energy transfer from phycobilisomes to photosystem II could occur if the steady-state proportion of photosystem II-coupled phycobilisomes were sufficiently high. This is likely, given the dense packing of phycobilisomes and photosystem II on the membrane surface¹⁶. The phycobilisome rod elements obscure much of the cytoplasmic surface of the membrane^{15,16}. The diffusion of phycobilisomes may therefore be necessary to allow access to ribosomes and other cytoplasmic components involved in the synthesis, regulation and repair of the reaction centres. The turnover of photosystem II is unusually rapid, because the complex must be continuously repaired after photodamage^{17,18}. The diffusion of the phycobilisomes may also

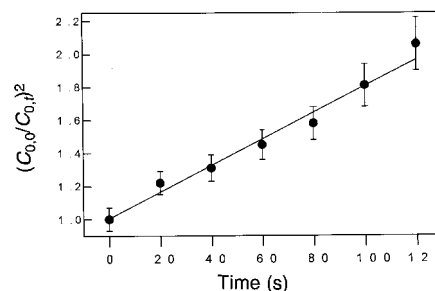


Figure 4 Calculation of the diffusion coefficient D for phycobilisomes from the time-dependence of the maximum bleach depth, $C_{y=0,t}$ in the measurement series shown in Figs 2a and 3a. A plot of $(C_{y=0,t=0}/C_{y=0,t})^2$ against time should give a straight line with gradient $8D/R_0^2$ where R_0 is the initial half-width $(1/e)^2$ of the bleach (see Box 1). In this case, R_0^2 was $6.3 \pm 0.3 \mu\text{m}^2$; weighted linear regression gives a plot gradient of $0.0080 \pm 0.0005 \text{ s}^{-1}$, so the diffusion coefficient D is calculated as $(6.3 \pm 0.5) \times 10^{-11} \text{ cm}^2 \text{s}^{-1}$.

be essential for state 1–state 2 transitions, which regulate energy transfer from phycobilisomes to photosystem II and photosystem I over a timescale of seconds to minutes^{14,19}. State transitions have been proposed to involve the decoupling of phycobilisomes from photosystem II and binding to photosystem I^{14,19–21}. Our results suggest that the phycobilisomes are the mobile element. A calculation based on our measured diffusion coefficients indicates that the diffusion of phycobilisomes from photosystem II to photosystem I could be complete within 100 ms. The rate of state transitions could be limited by the signal transduction pathway rather than the diffusion of the phycobilisomes. □

Methods

Growth and preparation of cells. *Dactylococcopsis salina*⁸ was grown at 30 °C and with moderate illumination in liquid growth medium⁸. Cells were spread on 0.6% agar containing the same growth medium, covered with a 0.2-mm quartz coverslip and placed on a stage heated to 30 °C under the microscope objective. The objective was immersed in a drop of glycerol on the coverslip.

FRAP measurement. We used the scanning confocal microscope Syclops²² at the Daresbury Laboratory. The lasers used were He–Ne (633 nm, 150 mW) or He–Cd (442 nm, 60 mW). The laser light was focused, passed through a 15-μm pinhole and focused onto the sample with a 100× objective lens. The vertical (Z) resolution was 2.6 μm (FWHM) and the resolution in the x–y plane was about 0.32 μm with 633-nm light, and 0.23 μm with 442 nm light (FWHM). The laser was scanned over the sample with oscillating mirrors. Fluorescence from the sample was separated from the excitation light with a polarizing beamsplitter and long-pass filters (Schott RG665), passed through a 40-μm pinhole and detected with a cooled photomultiplier (Hamamatsu R3896). The sample was bleached by scanning the laser repeatedly in one dimension across the cell for 20 s. The laser power was then reduced tenfold with a neutral density filter and the cell was imaged by scanning over a square of 75 × 75 μm in the x–y plane. There was no detectable photobleaching during the recording of successive image scans.

Data analysis. Fluorescence images were aligned and an image recorded before the bleach was subtracted. A one-dimensional bleaching profile was extracted (Fig. 1) using Optimas image analysis software (Optimas Corporation). To extract the width and depth of the bleach, the bleaching profile was fitted as a gaussian curve using Sigmaplot (Jandel Scientific).

Received 3 July; accepted 4 September 1997.

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Acknowledgements. We thank A. E. Walsby for discussion and for the culture of *Dactylococcopsis salina*.

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corrections

NMR structure of a receptor-bound G-protein peptide

Edward A. Dratz, Julie E. Furstenau, Christophe G. Lambert, Dennis L. Thireault, Helen Rarick, Theresa Schepers, Sergei Pakhlevanians & Heidi E. Hamm

Nature **363**, 276–280 (1993)

In this Letter we reversed the assignment of the amino proton resonances of 8HN (Cys 347) and 11HN (Phe 350), as pointed out by J. Russo and L. M. Gierasch (personal communication). This correction caused some alterations in the preferred structures at the same level of refinement as used in our Letter; however, further refinement has led to additional alterations in the receptor-bound structures. Some of the peptide-transferred NOESY NMR intensities showed evidence for mediation by rhodopsin protons at higher levels of structure refinement, as supported by spin-echo filtering of the rhodopsin magnetization during the Tr-NOESY mixing time. Protons on the large protein that are in intimate contact with the ligand can mediate ligand proton cross-relaxation and distort the apparent distances between ligand protons. Partial compensation for rhodopsin mediation of peptide Tr-NOESY, by spin-echo filtering, led to further alterations of the structures and considerable improvement of the agreement of the bound peptide structures with the data¹. More quantitative estimation of receptor mediation of peptide Tr-NOESY and refined structures of receptor-bound G-protein peptides will be published later. □

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DNA antisense therapy for asthma in an animal model

Jonathan W. Nyce & W. James Metzger

Nature **385**, 721–725 (1997)

It has come to our attention that this Letter contains the following errors, none of which changes the substance of the findings or the interpretation of the data. (1) The sequence given for the bradykinin B₂ mismatch molecule (B₂MM) contains a gap—it should read 5′-GGT GAT CTT GAG GAT TTC GGC-3′; (2) in the Methods section labelled “Receptor binding”, all dosages of antisense are indicated as 0.2 mg, whereas they should read 0.2 mg, 2.0 mg and 20.0 mg for each oligonucleotide tested; and (3) Scatchard plots of saturation isotherms were misdrawn; the correct K_d and B_{max} values are found in Table 1. □

Nucleic acid testing

DNA research techniques continue to proliferate and examples announced this week include tools for sequence analysis, DNA and RNA probes and cDNA panels for gene cloning.

24-hour sequencing service

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The service covers sequencing of 350–450-bp plasmids, PCR-fragments, M13 phages and phagemids, as well as 250–350-bp sequencing of lambda, cosmid, P1 and BAC clones. All of these are performed with a stated accuracy of >99 per cent and a turnaround time of 24 h, both guaranteed by GATC. If the samples arrive no later than noon, then the customer is said to receive the results within 24 h by e-mail or fax. If potential customers would like to test the sequencing service, GATC offers test sequencing free of charge.

Reader Enquiry No. 100

ThermoFidelase I for DNA sequencing

From Fidelity Systems

Automated sequencing of genomic DNA without cloning or amplifying beforehand

Derived from a highly stable thermostable DNA topoisomerase, this enzyme is designed to simplify DNA sequencing operations. When added to a sequencing cycle reactions, even samples that cannot be cloned and cannot be amplified by PCR can now be sequenced directly from bacterial genomic DNA, according to Fidelity Systems.

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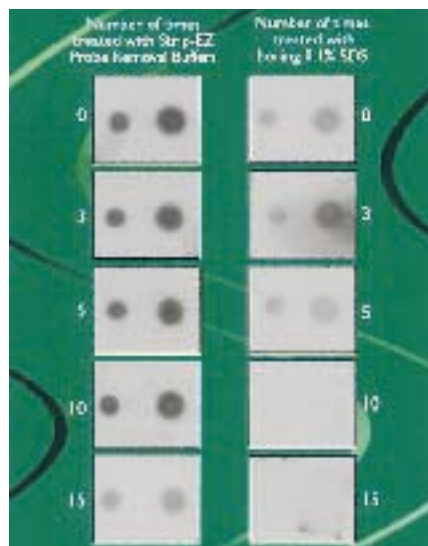
GeneQuest

From DNASTar

A tool for comprehensive analysis, organization and display of sequence information

This desktop software product offers a user friendly graphical interface to locate features of a sequence, perform BLAST searches and display features at scales from a whole chromosome down to the single nucleotide. The software finds ORFs, stops and starts, and identifies potential coding regions using Borodovsky's Markov method. Motifs, such as transcription factor binding sites and user-specified patterns, are readily explored alongside sites for splicing and polyadenylation. GeneQuest is designed to identify dyed, direct- and inverted-repeat sequences quickly, as well as displaying graphs of base composition and base skew, helping to elucidate the global structural organization. A web browser can then be launched from within the application to gather additional information on search hits from the target database.

Reader Enquiry No. 102



The 'full monty'? Not quite, but Ambion does have Strip-EZ for removal of RNA probes.

Probing the genetic material

Strip-EZ StripAble

From Ambion

RNA probes that wash well and remain stable under hybridization conditions

The buffer that this system uses allows the removal of the RNA probe from a blot with a simple 65 °C wash and a 10-min protocol. Degradation is specific to the Strip-EZ RNA probe because cleavage occurs only at a modified nucleotide that is incorporated during synthesis. The mild removal conditions extend the useful life of the blots and sample RNA, allowing up to ten stripping and rehybridization cycles for a single blot. The Strip-EZ StripAble RNA probe synthesis kit provides all the reagents necessary to produce and remove modified RNA probes, which can be used in northern, Southern, and related nucleic acid blot applications.

Reader Enquiry No. 103

Dual-colour haematology DNA probes

From Vysis

Research assays for identifying bcr/abl and TEL/AML1 gene fusions found in leukaemia

Two new probes have been created to identify chromosomal abnormalities found in leukaemia. The LSI bcr/abl ES dual-colour translocation probe is designed for the study of chronic myelogenous leukaemia (CML), whereas the LSI TEL/AML1 ES dual-colour translocation probe is intended for the investigation of childhood B-cell acute lymphocytic leukaemia (ALL).

Reader Enquiry No. 104

SignaTect

From Promega UK

A DNA-dependent protein kinase assay

The system allows for the measurement of DNA-dependent protein kinase (DNA-PK) in nuclear cell extracts or purified form. The assay is based on the streptavidin membrane technology, SAM², present in other SignaTect kinase assay systems. This technology is said to allow extremely low signal-to-noise ratios to be achieved through the use of DNA-PK-specific, biotinylated-peptide substrate, which becomes phosphorylated with radio-labelled phosphate in the presence of DNA-PK and radiolabelled ATP. The radio-labelled, biotinylated substrate is selectively captured on the streptavidin membrane. The kit includes a biotinylated, p53-derived peptide substrate (tumour suppressor protein), as well as all other reagents necessary for 96 assays.

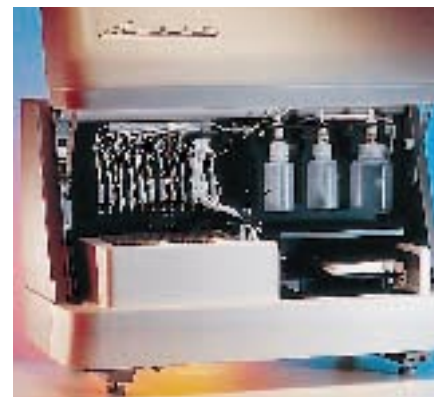
Reader Enquiry No. 105

Daras

From Tepnel Life Sciences

A new automated system for detecting nucleic acids that uses a specific oligonucleotide probe to capture the DNA fragment of interest

The system can be used to bind any specific PCR product, allowing non-complementary or misamplified DNA simply to be washed away. It can also clean up samples before amplification. Daras is fully automated and uses a microbead that carries the covalently linked oligonucleotide probe. Each capture particle is stated to carry billions of capture probes on its surface and virtually any complementary oligo-nucleotide can be covalently bound to the activated microbeads. The DNA capture particles are loaded into a small reaction column, which is capable of specifically binding up to 10¹⁴ copies of target DNA. The system can detect as little as



Tepnel's Daras automated DNA detection system.

25 pg of DNA fragments, says Tepnel. Complete sample handling and fluidics, temperature control and precise optical measurements are provided.

Reader Enquiry No. 106

Miscellaneous

In situ β -gal staining

From Stratagene

A staining kit for rapid and reliable in situ detection of β -galactosidase activity in transfected cells

Featuring a premixed staining reagent and a shorter protocol, the staining reaction produces a blue colour in transfected cells and can be performed directly in a tissue culture dish. According to the manufacturer, maximum signal can be produced in as little as 30–60 min with low background. A β -galactosidase plasmid is provided with the kit, together with all of the reagents needed to fix and stain transfection cells.

Reader Enquiry No. 107

IsoCode Stix

From Schleicher & Schuell

New blood collection dipsticks for isolation of DNA before performing PCR

High-quality DNA can be isolated in about 30 min with what is stated to be an inexpensive, reproducible protocol. Using a match-book-like configuration, which contains four dipsticks, each with a perforated, triangular end that serves to collect 10–12 pl of sample. Upon elution, blood components that inhibit amplification, such as haemoglobin, remain on the matrix when the DNA is released. Eluted DNA is suitable for both standard and long-range PCR. IsoCode Stix can be used with heparin- and EDTA-treated blood. Moreover, eight PCR reactions can be processed from one match-book, enough for immediate, confirmatory and archival purposes.

Reader Enquiry No. 108

fluoroDD kit

From Genomix

A kit that provides a fluorescent alternative to radioactive detection in differential display

For use with the genomixLRS DNA sequencing system, this new kit works with any of the company's five Hieroglyph mRNA profile kits for differential display. With the fluoroDD kit, the researcher can progress from RNAs to differential band patterns in a stated two days, cutting as much as a full day from the usual time requirement. Additionally, two complete gels can be run on one system within a 24-h period, essentially doubling the throughput. By eliminating the use of radioactive labelling, this system is safer and less expensive than other methods while providing comparable sensitivity and eliminat-



A bright future: a fluorescent alternative to radioactive detection, Genomix's fluoroDD kit.

ing the use of hazardous and costly radioactive labels. The fluoroDD kit uses fluorescent primers labelled with TMR.

Reader Enquiry No. 109

Rapid-Screen cDNA library panels

From OriGene Technologies

A product for cloning genes from cDNA libraries

This system is designed to allow you to complete an entire library screen, identify the cDNA clones for the gene(s) of interest and have plasmid DNA ready for transfection and expression studies in tissue culture cells in just three PCR amplifications. The master-plate for each library panel contains DNA from 500,000 cDNA clones and is screened in one set of 96-well PCR reactions. This first set of reactions identifies the subplates containing the clones of interest. The subplate are *Escherichia coli* glycerol stocks of the master-plate DNA, contained in standard 96-well microtitre dishes. Each plate contains 50 cDNA clones per well, with approximately 5,000 colonies per plate. The sub-plates are screened with a second set of 96-well polymerase chain reactions to identify the positive wells on the subplate. Then the cells from that subplate well are grown on bacterial agar plates and screened by a third round of PCR. The positive colonies identify the clone. Most importantly, the panels are generated with a bias towards longer cDNAs, and are well suited for identifying large clones and the previously hard-to-clone 5' end of cDNAs. These panels come from different tissues, including human placenta, spleen and fetal brain and fetal mouse.

Reader Enquiry No. 110

PCR product brochure

From Sigma

A 24-page brochure that features an entire line of PCR products

This literature includes enzymes, reagents, equipment, kits and accessories. Among the new products offered by Sigma are those for use in Long and Accurate PCR, hot-start PCR, standard PCR and PCR cloning.

Reader Enquiry No. 111

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